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(54) Title: IMPROVED PLASMID VECTORS FOR CELLULAR SLIME MOULDS OF THE GENUS DICTYOSTELIUM

Applicant L

(57) Abstract

The present invention relates generally to the fields of molecular biology and the production of recombinant protein using cellular slime moulds of the genus *Dictyostelium*. Most particularly, the present invention relates to novel strains of the genus *Dictyostelium*, recombinant plasmids for use with strains of the genus *Dictyostelium*, and polypeptides which facilitate the extrachromosomal replication of such plasmids in strains of the genus *Dictyostelium*. In particular, the present invention provides a polypeptide which facilitates the extrachromosomal replication of a recombinant plasmid in *Dictyostelium* spp in which the recombinant plasmid includes an origin of replication derived from a Ddp2-like plasmid but which lacks functional genes for extrachromosomal replication in wild type *Dictyostelium* spp. The extrachromosomal replicating plasmid constructed in accordance with the present invention are suitable for carrying a wide variety of genes and promoter sequences for control production of recombinant proteins by the biotechnology industry.

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IMPROVED PLASMID VECTORS FOR CELLULAR SLIME MOULDS OF THE GENUS DICTYOSTELIUM

Field of the Invention

Th present invention relates generally to the fields of molecular biology and the production of recombinant proteins by the biotechnology industry. More particularly, the present invention relates to novel strains of the genus Dictyostelium, recombinant plasmid vectors for use with strains of the genus Dictyostelium, and polypeptides which facilitate the extrachromosomal replication of such plasmids in strains of the genus Dictyostelium. Such extrachromosomally replicating plasmids, constructed with the art disclosed in this invention, are suitable for carrying a wide variety of genes and promoter sequences for the controlled production of recombinant proteins by the biotechnology industry.

BACKGROUND ART

As is well known in the art, genetic information is encoded on double stranded DNA molecules according to the sequence of four nucleotides containing different bases, adenine (A), thymine (T), cytosine (C) and guanine (G). Blocks of DNA sequences flanking genes often control gene activity by binding regulatory proteins and acting as recognition signals for enzymes of the cells biosynthetic machinery. Thus each cell contains a web of regulatory molecules which, by binding to specific DNA sequences, control gene activity. Other DNA sequences have crucial functions related to the control of DNA synthesis and partitioning of DNA into separate cells during cell division. These functions must be present on every DNA molecule in every cell or the DNA will be lost within a few cell generations.

Plasmids are usually circular DNA molecules possessing DNA sequences allowing them to replicate independently from chromosomal DNA. The DNA sequence

block where the replication of plasmid DNA is initiated is commonly called the "origin of replication" and the ability to replicate independently from chromosomal DNA is referred to as "extrachromosomal" replication.

Molecular biologists have developed techniques for cutting DNA molecules into fragments using sequence specific restriction enzymes, purifying the fragments and rejoining them in a different order. If one of the fragments of DNA used contains an origin of replication from an E. coli plasmid, the DNA can be inserted (transformed) into E. coli where it will replicate as a plasmid and can be produced in relatively large quantities. These techniques mean that genes from one organism, for example a human gene, can be flanked by regulatory DNA sequences from another organism, for example the bacterium E. coli, causing the human gene to be active in E. coli under entirely different regulatory controls. If the plasmid in question is constructed to include a second origin of replication allowing replication in a separate host cell, for example a mouse cell line, the gene can easily be transferred to the second host cell. Such a plasmid containing origins of replication for more than one host is commonly called a "shuttle vector". Plasmids are usually constructed to contain selectable markers, which are usually genes that confer antibiotic resistance or a metabolic advantage on the host cell to allow cells containing the plasmid to be distinguished from cells that have not received any plasmid during the transformation. Selectable marker genes must be flanked by appropriate DNA sequences to permit gene activity in the required host cell. It is possible to insert a plasmid into a host cell where it will be unable to replicate and so the only cells that survive the selection procedure will be those with the plasmid inserted into the host's chromosomal DNA. Such a

plasmid without an appropriate origin of replication is called an "integrating plasmid".

A cell produces polypeptides and proteins by initially making a messenger RNA copy of the gene, a process called transcription which is under the control of the flanking DNA sequences as summarised above. The cellular biosynthetic machinery then reads (translates) the RNA sequence in three nucleotide groups called codons which specify the amino acids to be incorporated into the polypeptide chain. The genetic code and mechanism of protein synthesis is very similar in all organisms so molecular biology techniques can be used to construct plasmid vectors to produce recombinant proteins in many different host cells irrespective of the source of the original gene. However, different host cells may process the protein in different ways so it may, for example, be folded incorrectly or cleaved by protease enzymes. Most importantly, eukaryotic cells differ from bacteria by frequently linking further chemical structures onto their proteins, a process called "post-translational modification". The chemical structures linked to eukaryotic proteins may include several types of oligosaccharide chains, glycolipids, lipids, sulphate and phosphate groups, all of which may affect the physical and biological properties of the molecule. Common effects of these post-translational modifications include increased resistance to proteolysis, altered immunogenicity, altered in vivo clearance and uptake by different cell types.

Post-translational modifications frequently occur on proteins that are secreted from cells or are present on cell membranes. Such proteins include a wide variety of soluble proteins that mediate inter-cellular interactions, blood proteins and cell surface receptors and so are of considerable interest to the pharmaceutical industry as either the targets for drug research or for in vivo

administration as therapeutic drugs in their own right. Since post-translational modifications may substantially alter the biological activity of such proteins (for example, tissue plasminogen activator (Ezzell, 1988, Nature 333, 383)), it is a goal of the biotechnology industry to produce each protein with a range of different modifications, both those that occur naturally and new modifications such as truncated oligosaccharide chains. However, proteins with post-translational modifications can only be produced in eukaryotic hosts and only a few eukaryotes have been used industrially. Mammalian tissue culture, for example Chinese Hamster Ovary Cells, is usually able to produce proteins with post-translational modifications similar to the natural protein, but is very expensive since these cells frequently require serum components in their growth media, have a slow growth rate and are relatively difficult to grow in large fermentors. Consequently, simple eukaryotes such as insect cells infected with baculovirus or yeast cells have been used to produce proteins with some post-translational modifications at a considerably lower cost. However, no one host is suitable for all recombinant proteins or can produce more than a few of the wide range of desirable post-translational modifications.

Dictyostelium has some advantages as a host for the production of low cost recombinant proteins with post-translational modifications (reviewed by Glenn & Williams, 1988, Australian J. Biotech. 1(4), 46-56). These include the production of N-linked gycosylation indistinguishable from the mammalian "high mannose form" and a wide variety of other structures including phosphatidyl-inositol-glycan tails. It is possible to alter the post-translational modifications produced by Dictyostelium by either using a range of mutant cultures which produce altered glycan structures or by simply

harvesting the Dictyostelium cells at different stages of the life cycle. A considerable body of scientific literature is available on the culture and genetics of Dictyostelium (Spudich J. Ed. (1987) Methods in Cell Biology Vol. 28, Academic Press, London). Dictyostelium has a number of characteristics suitable for use in the production of recombinant proteins in fermenters since they grow rapidly (4-10 hour cell cycle) and reach high densities (around 50 million cells per ml) in a nutrient medium. For some purposes, the ability of Dictyostelium to grow on a lawn of bacteria on a simple nutrient medium provides a remarkably simple and cheap culture technique when compared with mammalian or event insect tissue culture.

Dictyostelium strains are known to posses at least thirteen different plasmids (Farrar & Williams (1988) Trends in Genetics 4,343-348), but only Ddp1, Ddp2 and pDG1 have been studied in detail. Plasmid pDG1 is very unstable when cloned in E. coli (Orii et al (1989) Nucleic Acids Research 17, 1395-1408) so most constructions of shuttle vectors have used sequences from either Ddp1 or Ddp2. Plasmid Ddp1 is 12.3 Kb in size, but Ahern et al (Nucleic Acids Research (1988) 16, 6825-6837) showed that a vector containing a selectable marker (G418) resistance and only 2.2 Kb of Ddp1 was able to replicate extrachromosomally in D. discoideum. However, but the copy number per cell of this truncated plasmids lowered from the 150 characteristic of the parent plasmid to only 10-15 copies per cell. It is probable that this low copy number plasmid may not segregate efficiently at cell division and so may be unstable in the absence of continuous selection with the antibiotic G418. Incorporation of additional Dictyostelium DNA into such plasmids based on the Ddp1 origin of replication prevents them being maintained extrachromosomally (Gurniak et al,

(1990) Current Genetics 17, 321-325.) so they are unsuitable for use in the biotechnology industry.

The practical application of plasmids constructed from sections of Ddp2 has been limited by technical difficulties. The majority of techniques used in molecular biology are designed for use in the bacterium E. coli so the manipulation of Dictyostelium DNA requires it to be cloned into a vector capable of replication in E. coli. Consequently, research on Ddp2 has concentrated on the construction of recombinant "shuttle vectors" containing sequences allowing replication in both E. coli and Dictyostelium spp. Plasmid pMUW111 illustrates a shuttle vector that the present inventors have constructed (Fig. 4), which contains a 4.139 Kb Hind III - ScaI restriction fragment of Ddp2. This is close to the minimum amount of Ddp2 which can maintain extrachromosomal replication in wild type strains of Dictyostelium. Leiting and Noegel (1988 Plasmid 20, 241-248) have used a similar 4.0 Kb fragment of Ddp2 with approximately 300bp deleted close to the Xho I restriction site to construct a 9.6 Kb shuttle vector called pnDe1. However, despite containing minimal sections for the extrachromosomal replication of Ddp2, both these shuttle vectors (pMUW111 and pn DE1) suffer from problems of instability when maintained in E. coli. This is consistent with the Ddp2 DNA containing sequences that are unstable in E. coli. This problem can be mitigated by the use of host strains which lack exo-nuclease I and have low plasmid copy number (eg strain CES 201), but such hosts frequently present problems in preparing sufficient plasmid DNA for gene cloning experiments and for transforming back into Dictyostelium.

The necessity of using pieces of Ddp2 DNA approximately 4 Kb long to construct shuttle vectors also raises problems with regard to the final size of the

plasmid. The shuttle vector must contain selectable markers for both hosts together with appropriate promoter and termination sequences. These sequences comprise nearly 50% of the siz of plasmids pMUW111 and pnDe1. In addition, to be of any practical use a shuttle vector must be capable of carrying additional DNA containing a gene to be expressed in Dictyostelium together with appropriate controlling sequences. These additional sequences are likely to amount to a minimum of at least 2 Kb of DNA, bringing the total plasmid size to around 12 kilobase pairs. Increasing the size of the plasmid to over 10 Kb decreases its stability, a factor of considerable importance for the commercial production of recombinant proteins where, in order to avoid contamination of the product, regulatory authorities do not permit the use the antibiotic selection to ensure plasmid maintenance while cells are grown for extended periods. A large plasmid also raises difficulties since fewer restriction enzymes will cut the plasmid at only one position, the most suitable sites for genetic manipulations.

Shuttle vectors capable of being easily manipulated in E. coli and transferred back into Dictyostelium spp. are an essential pre-requisite for realising the potential of Dictyostelium in biotechnology. The present inventors have discovered means by which such vectors containing sections of Ddp2 smaller than 4 Kb can be constructed.

The present inventors have elucidated the full nucleotide sequence of the plasmid Ddp2 and have determined that a portion of this sequence encodes a gene designated Rep. The present inventors have shown that the presence of a polypeptide encoded by the Rep gene is essential for extrachromosomal replication of the Ddp2 plasmid.

Disclosure of the Inven tion

Accordingly, in a first aspect the present inv ntion

consists in a polypeptide which facilitates the extrachromosomal replication of a recombinant plasmid in Dictyostelium Spp, the recombinant plasmid including an origin of replication derived from a Ddp2-like plasmid, but lacking functional genes for extrachromosomal replication in wild type Dictyostelium Spp.

In a preferred embodiment of this aspect of the present invention the recombinant plasmid includes an origin of replication derived from plasmid Ddp2.

In a preferred embodiment of this aspect of the present invention the polypeptide has an amino acid sequence substantially as shown in Figure 2.

In a further preferred embodiment of this aspect of the present invention the polypeptide is encoded by a DNA sequence substantially as shown in Figure 1 from nucleotide 2378 to nucleotide 5038.

As used herein the phrase "Ddp2-like plasmid" is intended to cover plasmids having similar structure and similar functional regions to plasmid Ddp2. One example of such a Ddp2-like plasmid is plasmid pDG1.

In a second aspect the present invention consists in a recombinant plasmid vector, said vector being characterised in that it includes an origin of replication derived from plasmid Ddp2 or plasmid pDG1 and that it lacks functional genes for extrachromosomal replication in wild type Dictyostelium.

In a third aspect the present invention consists in a recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1 to nucleotide 2436 or a subsection thereof, and lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

In a fourth aspect the present invention consists in a recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1153 to

nucleotide 1775 or a subsection thereof, and lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

In a fifth aspect the present invention consists in a recombinant plasmid vector containing the DNA sequence TGTCATGACA but lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

In a sixth aspect the present invention consists in a recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1 to nucleotide 3241 or a portion thereof and lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

It is presently preferred that the recombinant plasmid vector includes a heterologous DNA sequence(s) encoding a desired polypeptide, a promoter sequence(s) that controls the expression of the heterologous DNA sequence(s), and preferably a sequence(s) including a selectable marker.

In a preferred embodiment of the present invention the recombinant plasmid vector includes a DNA sequence encoding a polypeptide and regulatory sequences for secretion of the desired polypeptide.

In a further preferred embodiment of the present invention the recombinant plasmid vector includes an expression cassette comprising a promoter DNA sequence derived from the Dictyostelium Actin 15 gene, a DNA sequence encoding the secretion signal peptide sequence of the D19 gene which encodes the protein PsA and a DNA sequence for RNA polyadenylation signal derived from the Actin 15 gene.

In a further preferred embodiment of the present invention, the recombinant vector includes the sequence of plasmid pMUW102, plasmid pMUW130 or plasmid pMUW1530 and a

heterologous DNA sequence encoding a desired polypeptide together with DNA sequences enabling the expression of the sequence encoding the desired polypeptide.

In a seventh asp ct, the present invention consists in a recombinant strain of Dictyostelium, the recombinant strain being characterised in that the strain includes a gene encoding a polypeptide which facilitates the extrachromosomal replication of a recombinant plasmid, the recombinant plasmid including an origin or replication derived from plasmid Ddp2 but lacking the functional gene for extrachromosomal replication in wild type Dictyostelium.

In a preferred embodiment of the present invention the recombinant plasmid includes an origin of replication derived from plasmid Ddp2, and is more preferably the recombinant plasmid of one of the second to sixth aspects of the present invention.

The gene encoding the polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid may be present in a chromosome of the recombinant strain of Dictyostelium or carried on a second plasmid, the second plasmid lacking an origin of replication derived from Ddp2. It is, however, presently preferred that the gene encoding the polypeptide is carried on a chromosome.

It is presently preferred that the recombinant strain of Dictyostelium has included within a chromosome the Rep gene.

In a further preferred embodiment of the present invention the chromosome of the recombinant strain of Dictyostelium includes a sequence substantially as shown in Figure 1 from nucleotide 1885 to nucleotide 5292.

In a further preferred embodiment of the present invention the recombinant strain of Dictyostelium harbors a recombinant plasmid, the recombinant plasmid including

an origin of replication derived from plasmid Ddp2 or plasmid pDG1, and preferably a DNA sequence encoding a desired polypeptide together with a DNA sequence enabling the expression of the sequence encoding the desired polypeptide, but lacking functional genes for extrachromosomal replication in wild type Dictyostelium.

In an eighth aspect the present invention consists in a method of producing a desired polypeptide comprising the following steps:-

1. Transforming a recombinant strain of Dictyostelium with a recombinant plasmid vector including a DNA sequence encoding the desired polypeptide and sequences enabling the expression of the DNA sequence encoding the desired polypeptide;
2. Culturing the recombinant strain of Dictyostelium under conditions which allow the expression of the DNA sequence encoding the desired polypeptide and allowing the desired polypeptide to be produced either as a cell bound form or be secreted; and
3. Recovering the secreted desired polypeptide;

characterised in that the recombinant plasmid vector includes an origin of replication derived from plasmid Ddp2 but lacks the functional genes for extrachromosomal replication in wild type Dictyostelium; and

that the recombinant strain of Dictyostelium includes a gene encoding a polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid.

As used herein the phrase "cell bound form" is intended to cover proteins either internal to the cell or present on the cell membrane.

In a preferred embodiment of this aspect of the present invention the gene encoding the polypeptide which facilitates the extrachromosomal replication of the

recombinant plasmid is present in a chromosome of the recombinant strain. Alternatively the gene is carried on a second recombinant plasmid present in the recombinant strain.

In a ninth aspect the present invention consists in a DNA molecule which includes a nucleotide sequence which encodes a polypeptide and which is capable of transforming Dictyostelium strains such that recombinant plasmid vectors which include an origin of replication derived from a Ddp2-like plasmid, preferably plasmid Ddp2, are incapable of extrachromosomal replication in wild type Dictyostelium spp. are capable of extrachromosomal replication in the transformed Dictyostelium strain.

In a preferred embodiment of this aspect of the present invention the DNA molecule includes a sequence substantially as shown in Fig. 1 from nucleotide 2378 to nucleotide 5038, or part thereof.

As stated above, the present invention relates to the construction of extrachromosomal plasmid vectors for Dictyostelium using much smaller sections of the plasmid Ddp2 than has previously been possible. The present invention enables the construction of plasmid vectors containing an origin of replication derived from Ddp2 which can be encoded on a section of Ddp2 DNA of less than 3.0 Kb, but omit sections of Ddp2 DNA that contain genes for polypeptides essential for replication and preferably DNA sequences that are unstable when cloned in E. coli. The replication of such plasmids can be achieved by maintaining them in recombinant strains of Dictyostelium where the polypeptides required for plasmid replication are provided by genes inserted into the chromosomal DNA of the host cell or alternatively into another compatible plasmid vector. The present invention enables the production of a wide range of plasmid vectors which may be constructed using the techniques known in th art and

disclosed herein, including plasmids designed for the expression of recombinant protein products in Dictyostelium spp.

The present invention further comprises the use of these recombinant Dictyostelium strains for the maintenance of recombinant plasmids containing an origin of replication derived from Ddp2 but lacking functional genes for replication proteins. The maintenance of recombinant plasmids in hosts that have been genetically modified to supply polypeptides necessary for plasmid replication is likely to be a crucial factor in the production of recombinant proteins using Dictyostelium spp.

SHORT DESCRIPTION OF THE DRAWINGS

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and accompanying figures, in which:-

Figure 1 is the nucleotide sequence of the Dictyostelium plasmid Ddp2. The sequence of one strand of DNA is shown, numbered clockwise from the SalI restriction enzyme site. The position of the recognition sites of restriction enzymes SalI, HindIII, BglII, NdeI, ClaI, EcoRI, EcoRV, PstI, BclI, XbaI, XhoI, AccI, HindII and ScaI are indicated. START and STOP indicates the position of the first and last codons of the Rep gene respective.
KEY: A =Adenine. C =Cytosine. G =Guanine. T =Thymine;

Figure 2 is the amino acid sequence of the polypeptide encoded by the Rep gene as derived from the DNA sequence of plasmid Ddp2. The nucleotide sequence of the coding strand of the Rep gene, numbered clockwise from the cleavage site of the SalI restriction enzyme, is aligned with the amino acid sequence predicted from the standard genetic code.
KEY: A =Adenine. C =Cytosine. G =Guanine. T =Thymine.
a =Alanine. c =Cysteine. d =Aspartic acid.

e =Glutamic acid. f =Phenylalanine.
g =Glycine. h =Histidine. i =Isoleucine.
k =Lysine. l =Leucine. m =Methionine.
n =Asparagine. p =Proline.
q =Asparagine. r =Arginine. s =Serine.
t = Threonine. v =Valine. w =Tryptophan;

Figure 3 is a schematic representation of the major structural features of Ddp2 aligned with a map of the cleavage sites of some restriction enzymes;

Figure 4 is a schematic representation of the construction of plasmid pMUW111;

Figure 5 is a schematic representation of the construction of plasmid pMUW110;

Figure 6 is a schematic representation of the construction of plasmid pMUW102;

Figure 7 is a schematic representation of the construction of plasmid pMUW130;

Figure 8 is a schematic representation which summarizes the Ddp2 sequences used to construct plasmids pMUW111, pMUW102, pMUW110 and pMUW130;

Figure 9 is a schematic representation of the construction of the shuttle vectors pMUW1530 and pMUW1580;

Figure 10 is the nucleotide sequence of the shuttle vector pMUW1530. The sequence of one strand of DNA is shown, numbered anti-clockwise from the ClaI restriction enzyme site. The position of the recognition sites of restriction enzymes ClaI, ScaI, BamHI, BglII and NdeI are indicated.

KEY: A =Adenine. C =Cytosine. G =Guanine. T =Thymine;

Figure 11 is a schematic representation of the construction of the promoter and secretion signal sequence sections of an expression cassette in plasmid pMUW1594;

Figure 12 is a schematic representation of the cloning of the polyadenylation sequence from the Dictyostelium Actin 15 gene into plasmid pMUW1560;

Figure 13 is a schematic representation of the construction of the expression cassette in pMUW1621;

Figure 14 is a schematic representation of the construction of an expression vectors pMUW1630 and pMUW1633 by insertion of the expression cassette into the shuttle vector pMUW1580; and

Figure 15 is the nucleotide sequence of the expression vector pMUW1630. The sequence of one strand of DNA is shown, numbered anti-clockwise from the ClaI restriction enzyme site. The position of the recognition sites of restriction enzymes ClaI, ScaI, NsiI, HindIII, SmaI and KpnI are indicated. START indicates the position of the first codon of secretion signal peptide in the expression cassette.

KEY: A =Adenine. C =Cytosine. G =Guanine. T =Thymine.

Best Mode of Carrying Out the Invention

The present inventors have established for the first time the full nucleotide sequence of the Dictyostelium plasmid Ddp2 as shown in Figure 1. The nucleotide sequence has been numbered clockwise around the circular DNA molecule starting at the single cut site of the SalI restriction enzyme. Detailed examination of the DNA sequence of Ddp2 has allowed different functional regions of the plasmid to be distinguished, as shown in Figure 3, and regions likely to be unstable when cloned in E. coli. The elucidation of these different functional regions has allowed the present inventors to overcome a number of the technical problems that have hitherto limited the use of extrachromosomal vectors in Dictyostelium.

The DNA sequence of Ddp2 between nucleotide 2378 and 5038 encodes a gene referred to herein as Rep. This section of Ddp2 contains a large "open reading frame" where one of the six possible ways to read the triple nucleotide genetic code (known as codons) has a long region without any of the codons that act as stop signals

for protein translation. Such an "op n reading frame" considered along with flanking sequences that are similar to the promoter and poly-adenylation signals of previously described Dictyostelium genes (Kimmel & Firtel, 1982 In The Development of Dictyostelium discoideum, Academic Press, New York, pp234-324) is strong evidence that the Rep gene could be transcribed into RNA and translated into a polypeptide containing 887 amino acids with the sequence shown in Figure 2. Evidence supporting the view that the Rep gene is translated into a polypeptide comes from the inability of plasmids constructed with interruptions to the Rep gene, for example pMUW102, to replicate in wild type strains of Dictyostelium discoideum. The RNA and polypeptide product of the Rep gene has not yet been detected and it is believed to be produced in only low amounts to positively regulate the initiation of plasmid replication by the host enzymes that normally replicate chromosomal DNA. However, it should be appreciated that either the messenger RNA or the translated polypeptide derived from the Rep gene could be processed by the cellular biochemical machinery to produce one or more shorter polypeptides. It is also likely that the polypeptide also contains regions that act as negative regulators of plasmid copy number. None of these areas of uncertainty subtract from the basic discovery that at least part of the open reading frame encodes a polypeptide that is essential for the replication of Ddp2. This finding explains the previously established need for shuttle vectors to contain a large section of Ddp2 DNA since such vectors would need to contain both the origin of replication and an additional 2.66 kilobase pair Rep gene plus flanking control sequences.

Plasmid vectors based on Ddp2 need to contain DNA from the section of Ddp2 between the HindIII restriction enzyme site at 1153 base pairs and the BgIII restriction

enzyme site at 1885 base pairs.

This is demonstrated by the inability of plasmids that lack this section of DNA, for example pMUW110 (Figure 5), to replicate in wild type strains of Dictyostelium. Plasmid pMUW110 contains the complete Rep gene plus flanking sequences including the polyadenylation sequences and 483 nucleotides encompassing the promoter region. Thus pMUW110 contains the sequences required to produce the polypeptide required for replication, but lacks a functional origin of replication. Consequently, a Ddp2 origin of DNA replication or associated control sequences must lie before the BgIII restriction enzyme site at 1885 base pairs. This region of Ddp2 is present in plasmid pMUW102 which contains the section of Ddp2 between the HindIII restriction enzyme site at 1153 base pairs and the XhoI restriction enzyme site at 3242 base pairs using plasmid pMUW102 (figure 6), but plasmid pMUW102 lacks a functional Rep gene and so is unable to replicate in wild type strains of Dictyostelium. The presence of a functional origin of replication in plasmid pMUW102 is demonstrated by transforming it into Dictyostelium strains along with plasmid pMUW110 to provide the essential replication polypeptide from the Ddp2 Rep gene. The present inventors experimental results clearly show that plasmid pMUW110 is inserted into the chromosomal DNA to form a stable recombinant strain of Dictyostelium and, in the same cells, plasmid pMUW102 is stably maintained as an extrachromosomal plasmid. This demonstration of an extrachromosomal plasmid containing an origin of replication from plasmid Ddp2 and its maintenance in a Dictyostelium strain by virtue of chromosomal DNA containing the Rep gene encoding polypeptides essential for plasmid replication represents a significant technical advance. It is apparent to one skilled in the art that similar techniques can be utilised

for the construction of a diverse range of plasmid vectors for Dictyostelium.

It is relevant to briefly examine the mechanism for selecting cells that were successfully transformed with both pMUW102 and pMUW110. Both these vectors contain a selectable marker conferring resistance to the antibiotic G418, but other genes could be used to serve the same function. In fact the present inventors have developed another resistance gene bleomycin for use as a selectable marker in Dictyostelium. The G418 resistance gene is under the control of Dictyostelium actin 6 promoter and the actin 8 3' poly-adenylation signals to ensure that it is expressed in Dictyostelium cells to provide a method of selecting the few cells that take up the plasmid DNA. Plasmid pMUW110 which lacks an origin of replication can only be retained in those few cells where the plasmid becomes integrated into the chromosomal DNA. Any cells that are transformed with only plasmid pMUW102 can only be resistant to G418 if the plasmid becomes integrated into the chromosomal DNA since this plasmid cannot replicate without the polypeptide produced by the Rep gene. However, some of the cells that receive both plasmids can have the plasmid pMUW110 integrated into the chromosomal DNA in a manner that preserves the function of the Rep gene and so will be able to maintain multiple extrachromosomal copies of the plasmid pMUW102. Once the cells transformed with both plasmids pMUW102 and pMUW110 have been selected by resistance to G418 they may be stably maintained in the absence of the antibiotic.

Plasmid pMUW102 contains 2089 base pairs of Ddp2; a considerably smaller section of Ddp2 than previously known to be capable of extrachromosomal replication. This sequence has been substantially shortened by removing more of the Ddp2 DNA sequences that are not essential for the replication of plasmid pMUW102 in recombinant strains of

Dictyostelium. The results with plasmid pMUW130 confirms that all the DNA sequenc s nec ssary for stable extrachromosomal r plication at high copy number are contained in a 622 base pair HindIII-ClaI fragment of Ddp2. In the light of present knowledge as disclosed herein, it is also relatively simple to ascertain the essential sequences within the section of Ddp2 between the HindIII restriction enzyme site at 1153 base pairs and the ClaI restriction enzyme site at 1885 base pairs using standard molecular biology techniques such as deletions and insertions. Experiments to determine the minimum section of Ddp2 DNA sequence necessary for plasmid vector construction have been carried out. Several copies of a TGTCATGACA sequence are essential for the function of the Ddp2 origin of replication.

The use of smaller sections of Ddp2 for vector construction than previously possible allows the omission of some of the sequences likely to be responsible for plasmid instability in E. coli. Plasmid pMUW130 contains only one copy of sequences in the 501 base pair inverted repeat of Ddp2 and does not contain the long stretches of poly-adenine or poly-thymidine found between the end of the open reading frame and the SalI restriction enzyme site. Such inverted repeats and poly-adenine or poly-thymidine sequences are known to be unstable in E. coli. Plasmid pMUW130 also omits the (GATGAA)11 repeat found at the end of the Rep gene and which is also likely to be unstable in E. coli. Therefore, it appears that the smaller sections of Ddp2 used to construct plasmid vectors according to this invention have less of the problems of stability in E. coli than were previously encountered using larger segments of Ddp2 DNA.

The integrating plasmid pMUW110 contains all the information necessary for the controlled expression of the Ddp2 Rep gene required to maintain the copy number of

plasmid pMUW102. This control of plasmid copy number could not be predicted since there would be no direct linkage between the number of copies of the plasmid and the Rep gene as in the original plasmid. It is thought that this copy number control is probably achieved by an auto-regulatory mechanism where the product of the Rep gene represses further transcription from the Rep gene and so maintains a constant cellular concentration of the polypeptide that regulates plasmid replication. The localisation of the promoter sequences to the section of Ddp2 DNA between the BgIII restriction enzyme site and the start of the Rep gene, as disclosed herein, allows future experiments to determine the regulatory mechanisms governing the transcription of the open reading frame and control of plasmid copy number. It is anticipated that this approach will lead to experimental control of plasmid replication and copy number by suitable modification or duplication of the control sequences.

In the experiments described herein, the plasmid pMUW110 has been stably integrated into the Dictyostelium chromosomal DNA using the same selective marker, G418 resistance, as present on the extrachromosomal plasmid pMUW102. However, there would be advantages in using a different selective marker on the integrating vector from that used for the extrachromosomal plasmid. The present inventors have developed a thymidylate synthase gene as a second marker for selection in a Dictyostelium discoideum strain that is unable to synthesise thymidine (Chang et al, 1989, Nucleic Acids Research 17, 3655-3661). The thymidylate synthase selection has the advantage for biotechnological uses in that the selection is maintained in the absence of any antibiotic. Clearly any combination of selectable markers can be used on the integrating or extrachromosomal vectors, but the preferred combination is to have the thymidylate synthase marker on the

extrachromosomal plasmid and maintain it in the enzyme deficient Dictyostelium strain. This means that, without using any antibiotic selection, any host cell losing either the extrachromosomal plasmid or the functional integrated vector would be unable to grow since any cell losing the production of the polypeptide necessary for plasmid replication would also lose the functions encoded on the extrachromosomal plasmid.

Examples of the application of the invention have been demonstrated by the construction of a range of shuttle vectors and the production of a recombinant protein in Dictyostelium discoideum. The novel shuttle vectors pMUW1530, pMUW1570 and pMUW1580 incorporate the Ddp2 origin of replication on the 600 bp XbaI - ClaI fragment (1175 - 1775 bp) of Ddp2 into a small E. coli plasmid (pMUW1510) that contains close to the minimal amount of sequence from pBR322 required for replication in E. coli in order to reduce the potential for these sequences to adversely effect the function of the shuttle vector in D. discoideum. Other useful features of these shuttle vectors is that they contain very few sites for six base restriction enzymes, apart from single BamHI and ClaI sites in appropriate positions for the insertion of additional DNA without disrupting essential functions. Sequences that might be inserted into such sites include genes for the production of recombinant proteins or selective markers, promoter sequences to control gene function and signal sequences for the correct processing of messenger RNA molecules and the translated proteins. This is illustrated by the construction of a novel "expression cassette" suitable for the production and secretion of a recombinant proteins from Dictyostelium cells. This expression cassette contains the promoter from the D. discoideum actin 15 gene, a section of the D19 gene encoding a secr tion signal peptide, the polylinker

from the E. coli plasmid pGEM3Z (for insertion of genes for expression) and lastly the polyadenylation signal from the D. discoideum actin 15 g ne. However, it will be apparent to one skilled in the art that a wide range of similar constructs could be made for this purpose using DNA sequences from other genes or even completely synthetic sequences serving the same functions.

The applications of the shuttle vector based on the technology disclosed in this document was demonstrated by the production of a recombinant protein from an E. coli gene for enzyme B-glucuronidase from D. discoideum cells containing an expression vector constructed by inserting the expression cassette into the shuttle vector pMUW1580.

Plasmid Ddp2 is believed to be the first functionally characterized member of a new group of structurally and functionally similar plasmids. This new group of plasmids can be defined as all encoding a single polypeptide of 700-1000 amino acids which is essential for plasmid replication and which has sequence homologies with the Ddp2 Rep gene, indicating a common evolutionary origin. Further, the origin of replication of these plasmids is associated with one arm of an inverted repeat sequence that is distinct from the Rep gene. The inventors confidently predict that the techniques they have disclosed in this application can be used to construct further extrachromosomal plasmid vectors for use in the biotechnology industry starting from the functionally analogous regions of any of this broader group of "Ddp2 - like" plasmids.

The only other member of this "Ddp2-like" group of plasmids to have been sequenced to date is plasmid pDG1 isolated from a unidentified Dictyostelium species (Orii et al (1987) Nucleic Acids Res. 15,1097-1107). Plasmid pDG1 has a very similar structure to Ddp2, possessing similar sized inverted repeats and a single open reading

frame analogous to the Rep gene of Ddp2. Despite plasmid pDG1 having been fully sequenced, nothing is known regarding the functions of these features or the location of the origin of replication (Orii et al (1989) Nucleic Acids Res. 17,1395-1408). The only recombinant shuttle vector produced with pDG1 sequences incorporated the long, 4.2 Kb ClaI fragment of pDG1, i.e., omitting only 0.2 Kb from the whole plasmid (Orii et al (1989) Nucleic Acids Res. 17,1395-1408). Such pDG1 based plasmids are very unstable in E. coli (Saing et al (1988) Mol. Gen. Genet. 214,1-5) and so are unsuitable for use in the production of recombinant proteins.

The plasmid pDG1 is recognized as a member of the "Ddp2-like" group of plasmids by virtue of its having a similar structure and having sequence homologies with Ddp2 in the region of the open reading frame at both the DNA and amino acid levels. The non-coding regions of these two plasmids have little sequence homology, apparently being free to diverge in the course of evolution. The presence of large inverted repeats in both pDG1 and Ddp2 is probably not a key feature of the group of "Ddp2-like" plasmids as only one copy is essential for the replication of Ddp2.

In the light of the functional data from the analogous regions of Ddp2, as disclosed in this application it is possible to re-evaluate the pDG1 sequence data and predict that pDG1 origin of replication lies outside the operating reading frame and overlaps with one of the inverted repeats. In addition, the speculation (Ori et al (1989) Nucleic Acids Res. 17,1395-1408) concerning the weak homologies of the Rep gene with reverse transcriptase is unlikely to be correct as the homology is not conserved in Ddp2. The Rep gene of Ddp2 can be aligned with the open reading frame of pDG1 with 35% of amino acids in identical positions indicating

considerable evolutionary homologies. The proteins encoded by the two plasmids also have similar structures, being comprised of two similar sized domains separated by a threonine rich sequence and the carboxy terminus of both proteins being a highly acidic glutamic and aspartic acid rich sequence. To one skilled in the art, the similarities between the proteins produced by these two plasmids indicates they have very similar functions and also indicates regions of high sequence homology which are most likely to have roles crucial for the proteins function. Whilst it is unlikely that the protein from pDG1 would be sufficient to cause replication of the Ddp2 origin of replication (and vice versa) because the sequence recognized by the protein is likely to be specific to the individual origin of replication, it is very likely that novel proteins constructed from sections of both proteins would function correctly. For example, the replacement of the acidic carboxy terminus of the Ddp2 Rep protein with the carboxy terminus of the pDG1 protein should not affect the ability of the molecule to allow replication from the Ddp2 origin of replication. Furthermore, it should be possible to change the specificity of the Ddp2 Rep gene simply by replacing the section of the protein that recognizes the Ddp2 origin of replication by a section recognizing an origin of replication from another member of the "Ddp2-like" group of plasmids. Clearly, the basic technology disclosed in this application, whereby, the replication protein and the origin of replication are separated onto separate vectors, is capable of a wide range of different applications for the construction of plasmid vectors incorporating sections from the broad group of "Ddp2-like" plasmids.

Example 1

Sequencing of plasmid Ddp2

Our laboratory at Macquarie University sequenced Ddp2

by cutting Ddp2 DNA into many small fragments and cloning them separately into a commercially available plasmid called pGEM3Z (Promega Corporation, Madison, USA). In this vector, small sections of Ddp2 DNA were stable and could be sequenced using a technique called "double stranded sequencing" where a small oligonucleotide is used to prime the synthesis of a new radio-labelled DNA strand on a template of denatured plasmid DNA. The oligonucleotide primer can be the complementary sequence to the SP6 or T7 regions flanking the cloning site or it can be a custom synthesised oligonucleotide with a sequence that matches part of the cloned Ddp2 DNA.

Ddp2 DNA was digested with the restriction enzymes ClaI, Sau3A, AluI or RsaI and cloned into the plasmid pGEM3Z at the AccI, BamHI or SmaI restriction enzyme sites using standard molecular biology techniques, and transformed into the E. coli strain JM109. Clones containing Ddp2 DNA were selected at random and stored in broth containing 15% glycerol and stored at -80 degrees.

Plasmid DNA from the clones was prepared using alkaline lysis and a RNAse enzyme treatment as recommended by the Promega literature on pGEM3Z. Before use in the sequencing reaction, 4ug of each plasmid was alkaline denatured with a brief treatment with 0.4M sodium hydroxide, precipitated with ethanol and annealed with 10 picomoles of oligonucleotide primer according to the procedure recommended by Pharmacia LKB Biotechnology (Uppsala, Sweden) for their T7 DNA polymerase sequencing kit which was used for the sequencing reaction. The sequencing reaction used ATP radio-labelled with ^{35}S. The radio-labelled DNA was separated on 6% acrylamide/8M urea gels which were then fixed in 10% methanol plus 10% acetic acid, dried and autoradiographed. The sequence revealed by the autoradiography films were entered into a computer and then overlapping sequences matched

automatically and compiled into the complete DNA sequence of Ddp2.

The full sequence of Ddp2 is available from the EMBL data base, accession number X51478.

Example 2

Location of the Origin of Replication of Ddp2

In further experiments the Ddp2 origin of replication was located to within the HindIII - ClaI fragment (1153-1775 bp) of Ddp2 as in plasmid pMUW130.

pMUW111

The plasmid pMUW111 was constructed by inserting the 4.1 Kb HindIII to ScaI fragment of Ddp2 into the SalI site of BIOSX. BIOSX is an integrating D.discoideum/E. coli shuttle vector constructed by Nellen et al. (Gene. 39 (1985) 155-163) and contains the Ampicillin and Kanamycin/G418 antibiotic resistance genes.

Ddp2 plasmid was first digested with restriction enzymes HindIII and ScaI. After the digestion was completed, the Hind III 5' overhang ends were made blunt using an end-filling reaction involving the enzyme DNA polymerase I "Klenow fragment". After this reaction was completed, it was fractionated in a 0.8% TBE agarose gel. The 4.1 Kb fragment was then excised from the gel and purified using a commercial kit, "Gene-Clean" (BIO101,Inc., USA). The purified DNA was then ligated with BIOSX that had been digested with SalI and end-filled. After ligation, the mixture was transformed into E. coli strain CES201 (Leach, D.R.F. and Stahl, F.W. (1983). Nature 305, 448-451). CES201 was made competent for transformation using the procedure as published by Hanahan, D. (J. Mol. Biol. (1983) 166, 557-580). The transformation mixture was then plated onto Luria-agar containing 50ug/ml ampicillin. E. coli ampicillin resistance transformants containing pMUW111 were confirmed by restriction fragment mapping of isolated plasmids and

also by radioactive hybridization using Ddp2 as a prob .

10 ug of pMUW111 was then used to transform Dictyostelium axenic strain, AX3K, using the standard calcium phosphate precipitation procedure developed by Nellen W. et al. (Mol. Cell. Biol. (1984) 4, 2890-2898) with G418 selection. To determine if pMUW111 was capable of autonomous replication, total nuclear DNA was isolated from G418 resistant transformants and then screened on a "lysis in the gel" as described by Noegel A. et al (J. Mol. Biol. (1985) 185, 447-450). The gel was then southern-transferred onto Zeta-probe blotting membrane (Bio-RAD) and hybridized using ^{32}P-labelled Ddp2 DNA. Autoradiography showed that pMUW111 had a higher mobility than the bulk chromosomal DNA, indicating it existed as an autonomously replicating plasmid.

pMUW102

The plasmid pMUW102 was constructed by inserting the 3.2 Kb SalI to XhoI fragment of Ddp2 into the Sal I site of BIOSX. This fragment contained only part of the open reading frame. Hence a complete functional protein(s) would not be expected to be produced by this construct.

Ddp2 plasmid was first digested with restriction enzymes SalI and XhoI. The sample was then fractionated in a 0.8% TBE agarose gel. The 3.2 Kb fragment was then excised from the gel and purified using a commercial kit, "Gene-Clean". The purified DNA was then ligated with BIOSX that had been digested with SalI. After ligation, the mixture was transformed into competent E. coli strain CES201. The transformation mixture was then plated onto Luria-agar containing 50 ug/ml ampicillin. E. coli ampicillin resistant transformants containing pMUW102 were confirmed by restriction fragment mapping of isolated plasmids and also by radioactive hybridization using Ddp2 as a probe.

10ug of pMUW102 was then used to transform

D. discoideum axenic strain, AX3K, using standard calcium phosphat precipitation procedure with G418 selection. To determine the fate of pMUW102, total nuclear DNA was isolated from G418 resistant transformants and then screened on a "lysis in the gel". The gel was then southern-blotted onto Zeta-probe blotting membrane and hybridized using ^{32}p-labelled Ddp2 DNA. Autoradiography showed that pMUW102 had the same mobility as the bulk chromosomal DNA, indicating it had integrated into chromosomal DNA and it was not capable of existing as a free plasmid. This experiment demonstrated that an intact open reading frame is essential for existence as an autonomously replicating plasmid.

pMUW110

The plasmid pMUW110 was constructed by inserting the 3.4 Kb BglII to ScaI fragment of Ddp2 into the Sal I site of BIOSX. This fragment contained the whole open reading frame "Rep gene" and the 5′ and 3′ flanking sequences that control the production of protein(s) specified by the open reading frame.

Ddp2 plasmid was first digested with restriction enzymes ScaI and BglII. After the digestion was completed, the BglII 5′ overhang ends were made blunt using an end-filling reaction involving the enzyme DNA polymerase I "Klenow fragment". After this reaction was completed, the sample was fractionated in a 0.8% TBE agarose gel. The 3.4 Kb fragment was then excised from the gel and purified using a commercial kit, "Gene-Clean". The purified DNA was then ligated with BIOSX that had been digested with SalI and end-filled.

After ligation, the mixture was transformed into E. coli strain CES201 that had been made competent for transformation. The transformation mixture was then plated onto Luria-agar containing 50 ug/ml ampicillin. E. coli ampicillin resistant transformants containing

pMUW110 were confirmed by restriction fragment mapping of isolated plasmids and also by radioactive hybridization using Ddp2 as a probe.

10ug of pMUW110 was then used to transform D.discoideum axenic strain, AX3K, using standard calcium phosphate precipitation procedure with G418 selection. To determine the fate of pMUW110, total nuclear DNA was isolated from G418 resistant transformants and then screened on a "lysis in the gel". The gel was then southern-transferred onto Zeta-probe blotting membrane and hybridized using ^{32}P-labelled Ddp2 DNA. Autoradiography showed that pMUW110 had the same mobility as the bulk chromosomal DNA, indicating it had integrated into the chromosomal DNA and it was not capable of existing as a free plasmid.

The difference between pMUW111 and pMUW110 is that 732 nucleotides between the HindIII restriction enzyme site at 1153 base pairs and the BglII restriction enzyme site at 1885 base pairs is missing in pMUW110. Hence the inability of pMUW110 to exist as a plasmid in AX3K could be explained by one of the following:

i) The 732bp sequence contained part of the origin of replication (ORI) of the plasmid Ddp2.

ii) The 732bp sequence contained cis acting element(s) that control the production of protein(s) specified by the open reading frame.

The first explanation was found to be correct by a subsequent experiment involving the co-transformation of AX3K with both pMUW102 and pMUW110. Screening of the G418-resistant transformants revealed that pMUW102 had a higher mobility than the bulk chromosomal DNA. This proved that pMUW102 could exist as an extrachromosomal plasmid only in the presence of pMUW110, which contained the intact open reading frame and hence is capable of providing the transacting protein(s) required for pMUW102

to replicate as a plasmid.

pMUW130

The plasmid pMUW130 was constructed by inserting the 622 base pair HindIII to ClaI fragment from Ddp2 (ie 1153 base pair to 1775 base pair) into the commercial E. coli plasmid pGEM3Z (Promega Corporation, Madison, USA) which had been digested with AccI and HindIII restriction enzymes. The construction of the plasmid used the same procedure as that of pMUW102 (above) except that the E. coli strain used was HB101.

Plasmid pMUW130 contains most of the 732 base pairs sequence that are in plasmid pMUW102, but not in plasmid pMUW110 and which was thought to be required for extrachromosomal replication. An experiment where pMUW102 and pMUW110 were co-transformed into D. discoideum strain AX3K demonstrated that pMUW130 can replicate extrachromosomally in the presence of pMUW110 which has been integrated into the chromosomal DNA. This confirms that an origin of DNA replication is located on this small HindIII - ClaI fragment of Ddp2 DNA. At approximately 3.3 kilobase pairs of DNA, pMUW130 was substantially smaller than previous shuttle vectors that had been constructed for Dictyostelium spp.

The location of an origin of replication on the HindIII - ClaI fragment incorporated into plasmid pMUW130 raises interesting scientific questions as to whether the similar sequences that occur in the small HindIII fragment (66-1153 bp) are also capable of acting as an origin of replication. This was investigated by cloning the small HindIII fragment (66-1153 bp) into the Hind III site of plasmid B10SX to form plasmid pMUW105. However, plasmid pMUW105 was unable to replicate extrachromosomally when mixed with plasmid pMUW110 (to provide the Rep gene) and transformed into D. discoideum strain AX3K. The small HindIII fragment in pMUW105 contains an entire, near

perfect copy of th 501 bp inverted repeat sequenc that forms most of the Ddp2 origin of replication in plasmid pMUW130. So the failure of pMUW105 to replicate extrachromosomally demonstrates that either the sequences just outside the 501 bp inverted repeat are essential for replication or the 11 nucleotide substitutions between the two copies of the 501 bp inverted repeat have prevented the copy in the small HindIII fragment in pMUW105 from acting as the origin of replication. Both of these possibilities result in the absence of or changes to copies of the DNA sequence TTTTTTGTCATGACACTTTTTTTTTTTTGTCATGACA, one copy of which lies just outside the 501 bp inverted repeat in pMUW130 and while a second copy of which is altered in pMUW105. This sequence contains two copies of a 10 bp palindrome TGTCATGACA (i.e. the two halves are symmetrical, so the complementary DNA strand will have the same sequence in the opposite orientation). Such palindromic sequences are typical of many sites recognized by DNA binding proteins, which would be consistent with this sequence being important for regulation of the origin of replication.

The Ddp2 origin of replication in plasmid pMUW130 contains two copies of the above oligo T sequence, each of which containstwo palindromes. Deletion of one copy of the sequence by cutting out the HindIII - BglII restriction fragment (1153-1369 bp, numbered according to Ddp2) of plasmid pMUW130 produced plasmid pMUW138 which is unable to replicate extrachromosomally in D. discoideum, thus demonstrating the importance of this sequence for the function of the origin of replication. However, it is unlikely that this sequence is the actual origin of replication, which is believed to lie in flanking sequences.

Example 3

Construction of a Small Shuttle Vector

A list of oligonucleotide sequences used in vector constructions is shown in Table I.

Despite plasmid pMUW130 being a great improvement on all shuttle vectors previously available for *D. discoideum*, it has some drawbacks for use in the biotechnology industry. Plasmid pMUW130 contains a disrupted polylinker (concentrated region of restriction enzyme sites) and DNA sequences derived from the Lac operon and the parent pBR322 plasmid which are not required in a *Dictyostelium* vector.

Ideally, the restriction enzyme sites in an expression plasmid should be only in positions convenient for the manipulation of the gene to be expressed and the amount of unnecessary DNA should be minimized. Shuttle plasmid pMUW 1530 was designed specifically for the purpose of easy manipulation of inserted sequences. This plasmid contains the minimal sequences derived from pBR322 that allow replication in *E. coli* plus the ampicillin resistance selective marker. The "poison sequences" that are known to interfere with replication from the SV40 origin of replication (Lusky & Botchan (1981) Nature 293, 79-81.) and gene expression in mammalian cells (Peterson et al (1987) Mol. Cell. Biol. 7,1563-1567) were excluded, although as yet their influence on *D. discoideum* plasmids is unknown. Other features of the plasmid include the creation of two unique six base restriction sites (BamHI and ClaI) positions suitable for the insertion of expression cassettes or selective markers.

Table 1.

LIST OF OLIGONUCLEOTIDE SEQUENCES USED IN VECTOR CONSTRUCTION.

The sequence (5' to 3') of the oligonucleotides synthesised at Macquarie University is shown together with the approximate position of restriction enzyme cutting sites.

PCR primers for cloning the actin15 promotor

GA190. TGGCCAAGCTTAGATCTACAAATTAATTAATCCC
EaeI HindIII BglII

GA188. CCCGGGATGTTCACCATGCATTTTTATTTTTTA
SmaI/AvaI FokI NsiI

PCR primers for cloning the actin15 3' region

GA189. TGCCGGTACCTAAATCATGAATGAAAGTGCT
KpnI

GA186. CCCGGGAATTCAGATCTTTTCATGGAGATTGTAT
SmaI/AvaI EcoRI BglII

PCR primers for cloning the secretion signal from the D19 gene

GA187. GGGAAGCTTGGATGAATTCAAAAAATGAAATTCCAACAT
HindIII FokI EcoRI

GA182. CCCGGGTCGACCTGCTATTGCATTTGCATATGTTAA
SmaI/AvaI SalI BspmI NdeI

Linker inserted into NdeI site to complete secretion signal sequence

GA297. TACGCCAATGCATATGAAAGCT
NsiI HindIII
NdeI

GA296. TAAGCTTTCATATGCATTGGCG
HindIII NdeI NseI

PCR primers used to clone pGEM3Z origin of replication

GA181. GGGGTGGATCCGCTAGCCGCATCGATAGGTGGCACTTTTCGG
BamHI NheI ClaI

GA179. GGAGGGATCCAAAGGCCAGCAAAAGGCCAGCAAAAGGC
BamHI

Sequencing oligonucleotide for pMUW1410

GA220 GAAGCATTTATCAGGG

Linker used to clone the gene for B-glucuronidase

GA310 AATTCCCGGG
EcoRI SmaI

pMUW1410

Plasmid pMUW1410 is an E. coli plasmid which was made to be the basis for construction of a series of shuttle vectors, including pMUW1530.

Plasmid pMUW1410 was constructed using two synthetic oligonucleotides GA179 and GA181 as primers to amplify the required pGEM3Z sequence in a polymerase chain reaction (PCR). The two oligonucleotide primers were each designed as two sections, the 5′ end of the sequences containing restriction sites required for cloning and the 3′ end of the sequences specifically matching the sequence of the plasmid pGEM3Z. The 3′ ends of the oligonucleotide GA179 is the same as the pGEM3Z nucleotides 452-472 bp (Promega Corp. numbering system) while the 3′ end of oligonucleotide GA181 is complementary to pGEM3Z nucleotides 2254-2240 bp, i.e. they prime opposite strands of the pGEM3Z DNA during the PCR reaction.

The PCR reaction was carried out using 10ng of pGEM3Z cut with restriction enzyme PvuII to linearized the plasmid, 20pico moles of each oligonucleotide, 0.03 mM of each of the four deoxynuclotide triphosphates dATP, dTTP, dCTP and dGTP, Taq polymerase buffer (Biores) to a final volume of 50 ul and 1.25 units of Taq polymerase (Biores). The reaction was carried out for eight cycles using 120 second incubations at 95 degrees to denature, 50 degrees to anneal and 72 degrees for the extension reaction. The polymerase was removed from product of the PCR reaction by extracting with phenol, then chloroform and the DNA precipitated with ethanol at -20 degrees. The product of the PCR (which consisted of the pGEM3Z sequence 452-2254 bp flanked by the sequences of the two oligonucleotides GA179 and GA181) was then digested with the restriction enzyme BamHI to cleave the BamHI sites at the 5′ end of the two oligonucleotides, and then the enzyme removed by extraction with 50% phenol/chloroform,

chloroform and then the DNA was precipitated with three volumes of ethanol at -70 degrees. Finally, the DNA product of the PCR reaction was self ligated using the BamHI sticky ends to form intact plasmids and the plasmids transformed into the E. coli strain Dh5a(Bethesda Research Laboratories) by electroporation using the procedures recommended by Biorad, the manufacturer of the "Gene pulser" equipment. The transformed cells were spread onto LB agar containing 100 ug ampicillin per ml. E. coli clones resistant to ampicillin were selected, their plasmids (e.g. pMUW1410) prepared by alkaline lysis and checked for size and the desired pattern of restriction enzyme sites using agar electrophoresis.

The plasmid pMUW1410 was approximately 1.8 Kb in size as expected for the desired portion of pGEM3Z (452-2254 bP) containing the pBR332 origin of replication and the ampicillin gene. Indeed, the ability of the E. coli clone containing pMUW1410 to replicate on ampicillin agar means the plasmid must contain a functional origin of replication and the ampicillin resistance gene. pMUW1410 also contains restriction sites for ClaI, BamHI and NheI derived from the synthetic oligonucleotides. The sequence of the plasmid pMUW1410 in the region of the BamHI site was confirmed using a T7 polymerase sequencing kit (Pharmacia) and a synthetic oligonucleotide GA220 which is designed to anneal to the ampicillin gene (2149-2164 bp, pGEM3Z numbering) so that the sequencing reaction covers the sequence derived from the oligonucleotides GA179 and GA181. The sequencing reaction confirmed that the oligonucleotides GA179 and GA181 used to create pMUW1410 had in fact bound to the expected positions in pGEM3Z and excludes the possibility of errors due to miss-priming at any other position.

pMUW1530

Shuttle vector pMUW1530 was constructed by inserting the XbaI - ClaI fragment (1175-1775 bp) of Ddp2 containing the origin of replication into the NheI and ClaI sites of plasmid pMUW1410.

Plasmid pMUW1015 containing the large AluI (1155-3223 bp) fragment of Ddp2 was used as the source of the Ddp2 origin of replication. 10 ug of pMUW1015 was digested with XbaI and EcoRI restriction enzymes and a 1.2 Kb DNA fragment (i.e. 1175-2436 bp of Ddp2) isolated by agarose gel purification. The appropriate DNA band was excised from the electrophoresis gel and frozen to disrupt the gel matrix. The DNA was extracted using the centrifugation methods of Heery et al ((1990) TIG 6,173.) and then phenol/chloroform extracted and ethanol precipitated to remove traces of the ethidium bromide stain. The DNA was further digested with the ClaI restriction enzyme and the 0.6 Kb XbaI - ClaI
fragment (1175-1775 bp, Ddp2 numbering) gel purified as described above.

Plasmid pMUW1410 was digested with the restriction enzyme NheI and subsequently with enzyme ClaI, since the NheI site is too close to the ClaI site to cut efficiently after the ClaI enzyme has cut. The digestion was then dephosphorylated by adding 1/40th volume of 20% SDS, 1/6th volume of 1M Tris buffer pH 9.0 and then 1 unit of Calf intestinal alkaline phosphatase (Boehringer) and incubating at 37 degrees for one hour. The enzyme was then removed by extracting with 50% phenol/chloroform followed by chloroform extraction and then the DNA precipitated with ammonium acetate and two volumes of ethanol.

The XbaI - ClaI fragment from plasmid pMUW1015 (i.e. the Ddp2 origin of replication) prepared above was ligated into the plasmid pMUW1415 (cut with NheI and ClaI and

treated with alkaline phosphatase), transformed into the E. coli strain "Sure" (Statagene) and plated onto LB agar containing 100 ug ampicillin per ml. E. coli clones resistant to ampicillin were selected, their plasmids (e.g. pMUW1530) prepared by alkaline lysis and checked for size and the desired pattern of restriction enzyme sites using agar electrophoresis.

Plasmid pMUW1530 is a 2.4 Kb shuttle plasmid containing the Ddp2 origin of replication inserted into the NheI and ClaI sites of plasmid pMUW1410. Evidence confirming this includes the presence of the Bg1II and NdeI sites from the Ddp2 origin of replication at the expected distance from the BamHI and ClaI sites found in pMUW1410. pMUW1530 does not contain the XbaI or NheI restriction sites used for cloning since the compatible "sticky ends" were destroyed by the ligation.

5ug of pMUW1530 mixed with 5ug of plasmid pMUW110 was then used to transform D. discoideum axenic strain, AX3K, using the standard calcium phosphate precipitation procedure with G418 selection. G418 resistant transformants were screened by "lysis in a gel", southern blotting onto Zeta-probe membrane and probed with ^{32}P labelled pGEM3Z. This demonstrated the presence of an extrachromosomal plasmid with the size of plasmid pMUW1530 containing pGEM3Z DNA sequences.

pMUW1570

Shuttle vector pMUW1570 is the same as pMUW1530, but with the NdeI restriction site removed to allow NdeI to be used for the manipulation of genes cloned into the plasmid.

Plasmid pMUW1530 was digested with the NdeI restriction enzyme in 11 ul of 10mM Tris buffer pH 7.5, 10mM MgCl and 50mM NaCl. The ends of the DNA were then filled by simply adding 1 unit of T7 polymerase and 3ul of the "C long" mix of deoxynucleotides supplied with the Pharmacia T7 polymerase sequencing kit and incubating at

room temperature for five minutes. The plasmid was then religated by adding 2ul ligation buffer (Boehringer), adjusting the volume to 20ul by adding water and 1 unit of T4 ligase and then incubating at 4 degrees overnight. The religated plasmid was transformed into the E. coli strain "Sure" (Statagene) and plated onto LB agar containing 100 ug ampicillin per ml. E. coli clones resistant to ampicillin were selected, their plasmids (e.g. pMUW1570) prepared by alkaline lysis and checked for size and the absence of the NdeI restriction site.

pMUW1580

Shuttle vector as pMUW1580 is the same pMUW1570, but with the Bg1II restriction site removed to allow Bg1II to be used for the manipulation of genes cloned into the plasmid.

Plasmid pMUW1530 was digested with the NdeI restriction enzyme, end filled with T7 polymerase, self ligated and transformed into E. coli using the same procedures as for pMUW1570. E. coli clones resistant to ampicillin were selected, their plasmids (e.g. pMUW1580) prepared by alkaline lysis and checked for size and the absence of the Bg1II restriction site.

Plasmid pMUW1580 contains a second ClaI site created by end filling the Bg1II site. However, in most strains of E. coli this sequence is methylated so that the ClaI enzyme will not cut the new ClaI site.

5ug of pMUW1580 was mixed with 5 ug of plasmid pMUW110 and used to transform the D. discoideum axenic strain, AX3K, using the standard calcium phosphate precipitation procedure with G418 selection. G418 resistant transformants were screened by "lysis in a gel", southern blotting onto Zeta-probe membrane and probed with ^{32}P labelled pGEM3Z. This demonstrated an extrachromosomal plasmid with the size of plasmid pMUW1580 containing pGEM3Z DNA sequences. Thus, plasmid pMUW1580

is a small, 2.4 Kb shuttle vector containing the minimum number of six base r striction sites, which is particularly suitable for use in the construction of expression vectors.

Example 4

Construction of an Expression Cassette

An "expression cassette" is a single, easily cloned piece of DNA which contains in their correct relative positions all the sequences required to ensure expression of a gene and the correct processing of the messenger RNA and protein product. Usually the cassette contains a number of restriction sites (polylinker) behind the promoter in a good position for inserting the gene to be expressed. The use of a well designed expression cassette greatly facilitates the expression of a range of genes and is much preferred to the alternative of cloning all the necessary DNA sequences on an adhoc basis.

We have designed a novel expression cassette specifically for insertion into the BamHI site of the shuttle vectors pMUW1530, pMUW1570 and pMUW1580. The expression cassette is designed to minimize the amount of unnecessary DNA sequences and restriction sites. This was achieved by cloning the required control and signal sequences using PCR techniques to insert at key positions the restriction sites required for cloning, using sites that can be destroyed during the construction procedure. The cassette contains a promoter from the D. discoideum actin 15 gene, a sequence coding for a secretion signal peptide, a polylinker containing restriction sites allowing the insertion of genes for expression and a polyadenylation signal sequence from the D. discoideum actin 15 gene.

Each component section of the expression cassette was cloned separately and then assembled into the complete cassette inside the polylinker of pGEM3Z.

Cloning the Actin 15 Promoter, Plasmid pMUW1480

The actin 15 promot r was selected because it is well characterised and is known to be expressed at a relatively high level soon after the onset of starvation (Cohen et al (1986) EMBO J. 5,3361-3366). For the purpose of the production of recombinant proteins, this pattern of expression is desirable to avoid the protein being produced during active growth where the resulting metabolic drain may cause a selective advantage for any non-secreting mutants.

The two synthetic oligonucleotides GA190 and GA188 were used as primers to amplify the required actin 15 promoter sequence in a polymerase chain reaction (PCR). The two oligonucleotide primers were each designed as two sections, the 5′ end of the sequences containing restriction sites required for cloning and the 3′ end of the sequences specifically matching the sequence of the Actin 15 gene in plasmid pTS1 (Chang et al (1989) Nucleic Acids Res. 17,3655-3661). The 3′ ends of the oligonucleotide GA190 is the same as the promoter nucleotides between -247 and -230 (numbering back from A of the ATG start codon) while the 3′ end of oligonucleotide GA188 is complementary to nucleotides between +3 and -13, i.e. they prime opposite strands of the actin 15 DNA during the PCR reaction.

The PCR reaction was carried out using 30 ng of pTS1 cut with restriction enzymes PvuII and ScaI to ensure the plasmid is unable to replicate during later cloning steps, 20p moles of each oligonucleotide, 0.03 mM of each of the four deoxynucleotide triphosphates dATP, dTTP, dCTP and dGTP, Taq polymerase buffer (Biores) to a final volume of 50 ul and 1.25 units of Taq polymerase (Biores). The reaction was carried out for ten cycles using 120 second incubations at 95 degrees to denature, 40 degrees to anneal and 72 degrees for the extension reaction. At the

end of the PCR reaction, 1 unit of T4 polymerase was added and incubated at room temperature for 15 minutes to ensure the ends of the DNA were blunt. 20 ug of glycogen in 1ul (Boehringer) was added and 2 ul of acetate buffer (to aid precipitation of the small DNA fragments) before the polymerases were removed by extracting with 50% phenol in choroform, then chloroform and the DNA precipitated with three volumes of ethanol at -70 degrees.

The product of the PCR reaction (consisting of the Actin 15 promoter sequence between -247 and +3 relative to the start codon flanked by the sequences of the two oligonucleotides GA190 and GA188) was shown to have the expected size of approximately 300 bp by electrophoresis in 1.6% agarose against size markers (BRESA) of phage SPP-1 digested with the restriction enzyme EcoRI.

The DNA product of the PCR reaction was mixed with 100ng of pGEM3Z which had been cut with the restriction enzyme SmaI to create blunt ends. The mixture was ligated with 3 units of T4 ligase in ligation buffer for two hours at room temperature and then precipitated with ammonium acetate and two volumes of ethanol. The religated plasmids were transformed into the E. coli strain Dh5a (Bethesda Research Laboratories) by electroporation using the procedures recommended by Biorad, the manufacturer of the "gene pulser" equipment. The transformed cells were plated onto LB agar containing 100 ug ampicillin per ml, 0.5mM IPTG (isopropyl-B-d-thiogalactopyanoside) and 50ug X-Gal (5-bromo-4-chloro-3-indolyl-B-galactoside) per ml. E. coli clones resistant to ampicillin and producing large white colonies (indicating the plasmid has DNA inserted into the polylinker) were selected, their plasmids (e.g. pMUW1480) prepared by alkaline lysis and checked for size and the desired pattern of restriction enzyme sites using agar electrophoresis.

The plasmid pMUW1480 digested by the restriction

enzyme PvuII produced a fragment with approximately of 700 bp, comprised of 379 bp of pGEM3Z sequ nc s containing an approximately 300 bp insert, as expected for the desired actin 15 promoter (250 bp) flanked by the sequences derived from the synthetic oligonucleotides GA190 and GA188. pMUW1480 also contains restriction sites for HindIII, Bg1II, NsiI and FokI derived from the synthetic oligonucleotides. The identity of the promoter inserted into plasmid pMUW1480 was confirmed by sequencing using a T7 polymerase sequencing kit (Pharmacia) and commercially supplied oligonucleotides (Promega) which anneal to SP6 and T7 regions flanking the polylinker. The sequencing excludes any possibility of errors in the sequence.

Cloning a Sequence for a Secretion Signal, Plasmid pMUW1450

Secretion of a protein requires a signal sequence at the amino terminal end of the polypeptide. This signal peptide is the first part of the protein to be transcribed and causes the ribosome to bind to the endoplasmic reticulum membranes and feed the nascent polypeptide across the membrane into the lumen of endoplasmic reticulum. Subsequently, the signal peptide is cleaved from the rest of the protein.

The D. discoideum protein PsA possesses a 20 amino acid signal peptide which has characteristics typical of many eukaryotic signal peptides (Perlman & Halvorson (1983) J. Mol. Bio. 167,391-409) and so it should be a reliable signal to use of the secretion of recombinant proteins.

The two synthetic oligonucleotides GA187 and GA182 were used as primers in a PCR reaction to amplify the DNA sequence coding for the PsA signal peptide from the D19 gene encoding the PsA protein (Early et al (1988) Mol. Cell. Biol. 8,3458-3466). The methods used were the same as described for cloning the actin 15 promoter (see above). However, some difficulty occurred in cloning the

correct product of the PCR reaction.

The plasmid pMUW1450 gave the correct size fragment when digested by the restriction enzyme PvuII, but when the insert was sequenced it was found that the oligonucleotide GA182 had not annealed to the D19 DNA in the anticipated position at the 3' end of the signal sequence. The DNA cloned in plasmid pMUW1450 contained the first oligonucleotide GA187 in the correct position 5' to the D19 start codon, but the DNA sequence continued past the end of the signal peptide as far as the PvuII site near the center of the gene. Investigation of the reason for the failure of the oligonucleotide GA182 to prime the PCR reaction correctly established that this sequence forms a hair pin loop, so it was unlikely to be available for binding to the D19 gene.

An alternative approach to modifying the 3' end the DNA coding for the PsA signal peptide is described below.

<u>Fusion of the promoter with the D19 (PsA) gene, plasmid pMUW1545</u>

Plasmid pMUW1450 contains the restriction sites derived from oligonucleotide GA187 that are required for the promoter and D19 gene sequences to be fused. This required a three way ligation to force clone the two DNA fragments into the NdeI and HindIII sites of pGEM3Z.

The DNA fragments to be fused were prepared by cutting 5ug of plasmid pMUW1450 with the restriction enzymes NdeI and ScaI and then purifying the largest (1.8 Kb) DNA fragment containing the D19 sequences by gel purification as described previously. The NdeI cleavage site at the end of this fragment occurs within the D19 sequence coding for the signal peptide. The promoter sequence was prepared by cutting 5ug of plasmid pMUW1480 with the HindIII and EcoRI restriction enzymes, which cut the HindIII site in oligonucleotide GA190 derived sequence 5' to the promoter and the EcoRI site in the polylinker,

yielding a 0.3 Kb fragment which was then purified by gel electrophoresis. The DNA fragments containing the D19 and promoter fragments were mixed together and digested with the FokI restriction enzyme which creates compatible ends at the ATG start codons in both sequences. The FokI digested fragments were extracted with 50% phenol in chloroform, then chloroform and then precipitated with three volumes of ethanol at -70 degrees. The FokI fragments were ligated with 0.5ug of pGEM3Z which has been cut with HindIII and NdeI, treated with alkaline phosphatase and purified by gel electrophoresis as described for plasmid pMUW1410. The religated plasmids were transformed into the E. coli "Sure" strain as described above and plated onto LB agar containing 100 ug ampicillin per ml. E. coli clones resistant to ampicillin were selected, their plasmids (e.g. pMUW1545) prepared by alkaline lysis and checked for the presence of the Bg1II restriction site from the oligonucleotide GA190, and the absence of the NsiI restriction sites that should have been removed from both of the inserted fragments.

Construction of the Full Promoter/Signal Sequence, pMUW1594

In order to replace the 3' end of the D19 sequence coding for the signal peptide a synthetic DNA sequence was cloned into the NdeI restriction site of plasmid pMUW1545. The synthetic DNA sequence is composed of two synthetic oligonucleotides GA297 (+ve strand) and a complementary sequence GA296 (-ve stand) which anneal to form double stranded DNA with ends compatible with the NdeI restriction site. In designing these oligonucleotides, the opportunity was taken to change the DNA sequence to optimize the codon usage for highly expressed genes, remove the potential to form hair pin loops and to remove the NdeI restriction site used to insert the oligonucleotides, leaving a single NdeI site suitable for cloning at the signal peptide cleavage site.

The DNA sequence changes do not alter the encoded amino acid sequence of the signal peptide.

The oligonucleotide GA297 and GA296 were phosphorylated with T4 kinase. 50p moles of each oligonucleotides in 50ul "One-for-all" buffer (Pharmacia) and 2uM dATP was incubated with 20 units T4 ligase at 37 degrees for 30 minutes and then the enzyme destroyed by heating to 100 degrees.

Plasmid pMUW1545 was linearized with NdeI restriction enzyme and ligated with 1 p mole of phosphorylated oligonucleotides GA297 and GA296 using 0.5 units of T4 ligase at 4 degrees overnight. The religated plasmids were transformed into the E. coli strain "Sure" (Statagene) and after one hour incubation at 37 degrees the organisms were inoculated into 500 ml LB broth containing 100 ug ampicillin per ml. After being shaken for 18 hours at 37 degrees, the cells were harvested and the mixed population of plasmids purified by alkaline lysis. 5 ug of the resulting mixture of plasmids were digested with the Hind III restriction enzyme and an approximately 0.3 Kb fragment of DNA purified by gel electrophoresis as described above. This 0.3 Kb fragment of DNA could only come from plasmids that are cut in the polylinker and also have the synthetic DNA sequence (which contains a second HindIII site) inserted into the NdeI restriction site of pMUW1545. Thus, this 0.3Kb fragment contains the full promoter - signal sequence construct.

The 0.3 Kb promoter - signal sequence was ligated into pGEM3Z that had been cut with HindIII, treated with alkaline phospnatase and purified by gel electrophoresis. The religated plasmid were transformed into the E. coli strain "Sure" (Statagene) and plated onto LB agar containing 100 ug ampicillin per ml. E. coli clones resistant to ampicillin were selected and their plasmids (e.g. pMUW1594) prepared by alkaline lysis. Plasmids were

checked for size and the correct orientation of the promoter (i.e. 5′ to the polylinker) using the position of the Bg1II site 5′ to the promoter. Clones were further screened by T7 polymerase sequencing (Pharmacia) using oligonucleotide GA187 to check the orientation of the inserted synthetic DNA sequence. Plasmid pMUW1594 had the required promoter and signal sequence in frame with the pGEM3Z polylinker encoded lac operon sequences.

Cloning the Actin 15 Polyadenylation Signal, Plasmid pMUW1512

The two synthetic oligonucleotides GA189 and GA186 were used as primers to amplify the actin 15 polyadenylation sequence in a polymerase chain reaction (PCR). The two oligonucleotide primers were each designed as two sections, the 5′ end of the sequences containing restrictions sites required for cloning and the 3′ end of the sequences specially matching the sequence of the Actin 15 gene in plasmid pTS1 (Chang et al (1989) Nucleic Acids Res. 17,3655-3661). The 3′ end of the oligonucleotide GA189 is designed to bind at the stop codon of the actin 15 gene and has one extra base pair added to the original sequence in order to place stop codons in all three reading frames, while the 3′ end of oligonucleotide GA186 is complementary to the sequence approximately 305 bp 3′, immediately preceding a EcoRV restriction site. The oligonucleotide GA186 replaces the EcoRV restriction site with Bg1II and EcoRI site for use in cloning.

The PCR amplification of the polyadenylation sequence was carried out using the identical DNA preparations and methods to the cloning of the actin 15 promoter described above, apart from the use of a different pair of oligonucleotides and the transformation of the plasmids into the "Sure" strain of E. coli. The plasmids produced (e.g. pMUW1512) were digested by the restriction enzyme

PvuII and screened for the presence of a fragment of approximately 800 bp, comprised of 379 bp of pGEM3Z sequences containing an approximately 400 bp insert. The plasmids were further digested with the restriction enzymes Bg1II, EcoRI and KpnI (separately) to check for the presence of the restriction sites from the two oligonucleotides. Plasmids pMUW1512 and pMUW1515 (opposite orientations of the insert) were sequenced to confirm the polyadenylation signal contained no errors using a T7 polymerase sequencing kit (Pharmacia) and commercially supplied oligonucleotides (Promega) which anneal to SP6 and T7 regions flanking the polylinker.

5 ug of plasmid pMUW1512 was digested with KpnI and subsequently with EcoRI restriction enzymes and an approximately 0.4 Kb fragment containing the polyadenylation signal purified by gel electrophoresis as described previously. This 0.4Kb fragment was ligated into 1 ug of plasmid pGEM32 which was also digested with KpnI and EcoRI, treated with alkaline phosphatase and then purified by gel electrophoresis. The plasmids were transformed into E. coli strain "Sure" plated onto LB agar containing ampicillin as described previously. Plasmids (e.g. pMUW1560) from the ampicillin resistant clones were screened for the correct sized insert (0.4 Kb) and the presence of a Bg1II site derived from oligonucleotide GA186. Plasmid pMUW1560 contains the actin 15 polyadenylation signal in the correct position and orientation for the final expression cassette.

Construction of the Complete Expression Cassette, Plasmid pMUW1621

The expression cassette was completed in a single cloning step combining the fused promoter/signal sequence from plasmid pMUW1594 with the polyadenylation sequence from plasmid pMUW1560.

Plasmid pMUW1560 was digested with the restriction

enzymes SalI and ScaI and the smaller 1.2 kG Kb fragment containing the polyadenylation signal purified by gel electrophoresis as previously described. Plasmid pMUW1594 was also digested with SalI and ScaI enzymes and the larger 2 Kb fragment containing the promoter and signal sequence purified by gel electrophoresis. The two fragments were pooled, ligated and transformed into the "Sure" strain of E. coli. The identity of the isolated plasmids (e.g. pMUW1621) was confirmed by cutting with the restriction enzyme Bg1II to produce a 0.7 Kb fragment. This fragment can only be produced by plasmids containing two Bg1II sites, one derived for the oligonucleotide GA190 used to clone the promoter and the second derived from oligonucleotide GA186 used to clone the polyadenylation signal.

Insertion of the Expression Cassette into the Shuttle Vector

The shuttle vector pMUW1580 was linearized using restriction enzyme BamHI, treated with alkaline phosphatase and purified by gel electrophoresis as previously described. The expression cassette in the 0.7 Kb Bg1II fragment from plasmid pMUW1621 was also purified by gel electrophoresis and ligated into the linearized plasmid pMUW1580. The ends of the DNA fragments produced by the Bg1II and BamHI enzymes are compatible, so both restriction sites are destroyed in the ligation. The resulting plasmids produced in the E. coli "Sure" strain were digested with ClaI and HindIII enzymes to screen for the presence of the polylinker in the expression cassette and the orientation of the expression cassette in the plasmid. Plasmids pMUW1630 and 1633 had the opposite orientations of the expression cassette.

Insertion of the GUS Gene into the Expression Vector

The GUS gene is the E. coli gene for the enzyme B-glucuronidase which has been modified by the insertion

of SalI and NcoI restriction enzyme sites at the start codon of the gene, an EcoRI site at the 3' end of the gene and a BamHI site removed from the center of the gene (Jefferson et al (1986) PNAS 83, 8447). Plasmid pRAJ275 containing this construct was purchased from Clontech Laboratories Inc., California, USA.

In order that the GUS gene could be easily sequenced, it was inserted into pGEM3Z. The GUS gene was cut out of plasmid pRAJ175 with the restriction enzymes SalI and EcoRI, purified by gel electrophoresis and ligated into plasmid pGEM3Z which had been cut with the same enzymes, treated with alkaline phosphatase and gel purified. The plasmid with the GUS gene inserted was pMUW1550.

A SmaI restriction site was inserted into the EcoRI site of plasmid pMUW1550 using oligonucleotide GA310 as a linker. Oligonucleotide GA310 was phosphorylated as previously described in the section on the construction of the full promoter/signal sequence. 1 pmole of phosphorylated GA130 was mixed with 0.5 ug of plasmid pMUW1550 which had been cut with the EcoRI restriction enzyme and purified by gel electrophoresis. The mixture was ligated at 4 degrees overnight and then transformed into the E. coli "Sure" strain. The transformants were incubated for one hour in SOC medium and then inoculated into 50 ml of LB broth containing 100ug ampicillin per ml. After shaking at 37 degrees for 18 hours the cells were harvested, plasmids purified and cut with the SmaI restriction enzyme. Only the plasmids containing the oligonucleotide GA130 contain a SmaI site, so the linearized plasmids were purified by gel electrophoresis, religated and transformed back into the E. coli strain "Sure".

1 ug of plasmid pMUW1558 containing a the GUS gene with the SmaI restriction site inserted into the EcoRI site at the 3' end of the gene was cut with the

restriction enzymes SalI and SmaI and the 1.9Kb gene purified by gel electrophoresis. The polylinker in the expression vector pMUW1630 was also cut with the restriction enzymes SalI and SmaI, treated with alkaline phosphatase and purified by gel electrophoresis. The two purified DNA fragments were ligated, transformed into the "Sure" strain of E. coli and plasmids purified from ampicillin resistant clones. Plasmid pMUW1653 contained the GUS gene cloned in frame into the SalI site of the expression vector. This was confirmed by restriction mapping using the sites for NcoI and EcoRI enzymes at the 5' and 3' ends of the GUS Gene respectively. The region of the fusion between the sequence encoding the secretion signal and the 5' end of the GUS gene sequencing plasmid was confirmed by DNA sequencing using a T7 polymerase kit (Pharmacia) and oligonucleotide GA187.

Expression of the GUS gene in D. discoideum

The suitability of the expression vector for the expression of recombinant genes was confirmed by transforming 5 ug of the expression plasmid pMUW1653 (containing the E. coli B-glucuronidase gene) and 5ug of plasmid pMUW110 (containing the Ddp2 Rep gene and a G418 resistance marker) into D. discoideum strain AX3K using the calcium phosphate precipitation procedure described previously. After one week under G418 selection, the culture supernatant from the transformants was tested for the presence of the GUS enzyme activity using 1mM p-nitrophenyl-B-D glucuronide substrate in 50mM sodium phosphate pH7.0, 10mM 2- mercaptoethanol and 0.1% Triton X-100. A green colouration indicated the presence of the enzyme B-glucuronidase secreted from D. discoideum. Culture supernatants from cells transformed with the expression vector pMUW1630 did not contain B-glucuronidase.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made

to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. A polypeptide which facilitates the extrachromosomal replication of a recombinant plasmid in Dictyostelium spp, the recombinant plasmid including an origin of replication derived from a Ddp2-like plasmid but lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

2. A polypeptide as claimed in claim 1 in which the recombinant plasmid includes an origin of replication derived from plasmid Ddp2.

3. A polypeptide as claimed in claim 2 in which the polypeptide has an amino acid sequence substantially as shown in Figure 2.

4. A polypeptide as claimed in claim 2 in which the polypeptide is encoded by a DNA sequence substantially as shown in Figure 1 from nucleotide 2378 to nucleotide 5038.

5. A recombinant plasmid vector including an origin of replication derived from plasmid Ddp2 or plasmid pDG1 and lacking functional genes for extrachromosomal replication in wide type Dictyostelium spp.

6. A recombinant plasmid vector is claimed in claim 5 in which the vector includes an origin of replication derived from plasmid Ddp2.

7. A recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1 to nucleotide 2436 or a subsection thereof, and lacking functional genes for extrachromosomal replication in wide type Dictyostelium spp.

8. A recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1153 to nucleotide 1775 or a subsection thereof, and lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

9. A recombinant plasmid vector containing the DNA sequence TGTCATGACA but lacking functional genes for

extrachromosomal replication in wild type Dictyostelium spp.

10. A recombinant plasmid vector containing a DNA sequence substantially as shown in Figure 1 from nucleotide 1 to nucleotide 3241 or a portion thereof and lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

11. A recombinant plasmid vector as claimed in any one claims 5 to 10 in which the recombinant plasmid includes a heterologous DNA sequence(s) encoding a desired polypeptide together with a promoter sequence(s) that controls the expression of the heterologous DNA sequence(s).

12. A recombinant plasmid as claimed in claim 11 in which the plasmid includes a DNA sequence encoding a polypeptide signal for secretion of the desired polypeptide.

13. A recombinant plasmid vector as claimed in any one of claims 5 to 10 in which the recombinant plasmid vector includes an expression cassette comprising a promoter DNA sequence derived from Dictyostelium Actin 15 gene, a DNA sequence encoding the secretion signal peptide sequence of the D19 gene of the protein PsA and a DNA signal sequence for RNA polyadenylation derived from the Actin 15 gene.

14. A DNA molecule including a nucleotide sequence which encodes the polypeptide as claimed in any one of claims 1 to 3 and which is capable of transforming Dictyostelium strains such that the recombinant plasmid vectors as claimed in any one claims 5 to 13 are capable of extrachromosomal replication in the transformed Dictyostelium strain.

15. A DNA molecule as claimed in claim 14 in which the DNA molecule includes a sequence substantially as shown in Figure 1 from nucleotide 2378 to nucleotide 5038, or part thereof.

16. A recombinant strain of Dictyostelium spp in which

the recombinant strain includes a gene encoding a polypeptide which facilitates the extrachromosomal replication of a recombinant plasmid, the recombinant plasmid including an origin of replication derived from a Ddp 2-like plasmid but lacking functional genes for extrachromosomal replication in wild type Dictyostelium spp.

17. A recombinant strain of Dictyostelium as claimed in claim 16 in which the recombinant plasmid is as claimed in any one of claims 5 to 13.

18. A recombinant strain of Dictyostelium as claimed in claim 16 or 17 in which the gene encoding the polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid is present in a chromosome of the recombinant strain of Dictyostelium.

19. A recombinant strain of Dictyostelium as claimed in any one of claims 16 to 18 in which the gene encoding the polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid has a DNA sequence substantially as shown in Figure 1 from nucleotide 2378 to nucleotide 5038.

20. A recombinant strain of Dictyostelium as claimed in claim 18 in which the gene encoding the polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid has a DNA sequence substantially as shown in Figure 1 from nucleotide 1885 to nucleotide 5292.

21. A recombinant strain of Dictyostelium as claimed in any one of claims 15 to 20 in which the recombinant strain of Dictyostelium harbours a recombinant plasmid as claimed in any one of claims 5 to 13.

22. A method of producing a desired polypeptide comprising the following steps:-

1. Transforming a recombinant strain of Dictyostelium spp with a recombinant plasmid vector including a DNA sequence encoding the desired

polypeptide and sequences enabling the expression of the DNA sequence encoding the desired polypeptide;

2. Culturing the recombinant strain of Dictyostelium under conditions which allow the expression of the DNA sequence encoding the desired polypeptide and allowing the desired polypeptide to be produced either as a cell bound form or secreted;

3. Recovering the desired polypeptide;

characterised in that the recombinant plasmid vector includes an origin of replication derived from a Ddp2-like plasmid but lacks the functional genes for extrachromosomal replication in wild type Dictyostelium spp, and the recombinant strain of Dictyostelium includes a gene encoding a polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid.

23. A method as claimed in claim 22 in which the desired polypeptide is produced in a cell bound form.

24. A method as claimed in claim 22 in which the gene encoding the polypeptide which facilitates the extrachromosomal replication of the recombinant plasmid is present in the chromosome of the recombinant strain.

25. Recombinant plasmid vector pMUW102 as hereinbefore described.

26. Recombinant plasmid vector pMUW111 as hereinbefore described.

27. Recombinant plasmid vector pMUW110 as hereinbefore described.

28. Recombinant plasmid vector pMUW130 as hereinbefore described.

29. Recombinant plasmid vector pMUW1530 as hereinbefore described.

30. Recombinant plasmid vector pMUW1570 as hereinbefore described.

31. Recombinant plasmid vector pMUW1580 as hereinbefore described.

32. Recombinant plasmid vector pMUW1594 as hereinbefore described.

33. Recombinant plasmid vector pMUW1560 as hereinbefore described.

34. Recombinant plasmid vector pMUW1621 as hereinbefore described.

35. Recombinant plasmid vector pMUW1630 as hereinbefore described.

36. Recombinant plasmid vector pMUW1633 as hereinbefore described.

37. Recombinant plasmid vector pMUW1600 as hereinbefore described.

Fig. 1-1

SalI
TCGACAAATA TCAAGGGTTG GAATCTTGTA AAAATTTTCC CGTTATCGCA
10 20 30 40 50

HindIII
AACAATCAAA GTTTAAGCTT CAATCTTCAA TAATAATTTT AACTTTATCT
60 70 80 90 100

ClaI
CTTTCAATTT TAATAATTTT TTTCAAAAAT TGAAAATGGT ATAGATCGAT
110 120 130 140 150

AGATCACCTT TTTTAGAGAT AAACCATGAA AAAGACATAA AAAATAAAGG
160 170 180 190 200

TCATCAAAGT ATTAAAAAAA ATTAATTATC TTTTTAACTT TGAAAAAAAA
210 220 230 240 250

AAATAAAAAA AAATAAAAAA AAAAAATTCT TTGTTTTAAT AACTTTTAAA
260 270 280 290 300

ATTATTAAAA ATAGTATAGA TTTAAAGATC ACAATTTTTT ATAATTAACT
310 320 330 340 350

ACATAAAATT TATAAAAAAT GAGGGTCATG AAGATATATA AATAATTATT
360 370 380 390 400

TAATTATTAA ATATTTAATT ATTTATTTAA CTTAAAAAAA AAAAAAAGGA
410 420 430 440 450

AAAAAAGGAA AAAAAAAGTG AAAAAGGTGG GAAAATGAAA AAAAAAGTGA
460 470 480 490 500

AAAAAATGCC CAAAAAAATT TTTATATGAG AAAAAAATTA CGTAAAAAAA
510 520 530 540 550

AAATAAGTCT GACCCAAATC GAAAAATAAT AAAAGAGGGG AAAGTAATTA
560 570 580 590 600

TAACTAGGTT AGTTTTTTAT AATTTTTACA TATTTGTTAA TAACTTTTAA
610 620 630 640 650

NdeI
TTTTGAATCA TATGATATTA CATCGTCCCG TTGAAAAAAA AAAAAAAAAT
660 670 680 690 700

TTTTTTTTCA AACATTTTCA TTTTTTAAAA AATGATATAA AATTTTAAAC
710 720 730 740 750

TAAACTATTT TATTAAATAC AAATATATAA CTTTATCTTA ATCAATTTTT
760 770 780 790 800

BglII
TTGGTTTATA CATATTTATG TTCGTACTGA AGTATAGATC TTATTACTAA
810 820 830 840 850

AGTTTCAAAA GTTTTAAAAA AAATTAAAGG GGGTAAATAT ATAACTTTCT
860 870 880 890 900

Fig. 1-2

```
GTTTTTTTCA ATTCTGTCAT GACAGAAAGG TAAAAAGTGT CATGACAAAA
       910        920        930        940        950

AAAAAAAAAA AAAAAATTTA TTTCTTCAAT AGGTATTGAA ATGACCTCCG
       960        970        980        990       1000

TTTTTAATAA AAAGTATATA TTTGTGCTTT CCTAGATGAA ATAAGGTTAT
      1010       1020       1030       1040       1050

TTGAGCTTAA TTCAGATTAT TATAAGATTA TTATAAAAAA ATGAAAAACT
      1060       1070       1080       1090       1100

GTCATGACAG TTTTTGTAAG TTTCTTATAG TTTTTTTTAA TGATCTGAAT
      1110       1120       1130       1140       1150
                            XbaI
HindIII
TAAGCTTAAA TAACCTTATT TCATCTAGAC GAGCACAAAT ATATACTTTT
      1160       1170       1180       1190       1200

TATTAAAAAC GGAGGTCATT TCAATACCTA TTGAAGAAAT AAATTTTTTT
      1210       1220       1230       1240       1250

TTTTTTTTTT TTTGTCATGA CACTTTTTTT TTTTTGTCAT GACAGAATTG
      1260       1270       1280       1290       1300

AAAAAAACAG AAAGTTATAT ATTTACCCCC TTTAATTTTT TTTAAAACTT
      1310       1320       1330       1340       1350
                      BglII
TTGAAACTTT AGTAATAAGA TCTATACTTC AGTACGAACA TAAATATGTA
      1360       1370       1380       1390       1400

TAAACCAAAA AAATTGATTA AGATAAAGTT ATATGTTTGT ATTTAATAAA
      1410       1420       1430       1440       1450

ATAGTTTAGT TTAAAATTTT ATATCATTTT TTAAAAAATG AAAATGTTTG
      1460       1470       1480       1490       1500
                                                  NdeI
AAAAAAAAAA TTTTTTTTTT TTTTTTCAAC GGGACGATGT AATATCATAT
      1510       1520       1530       1540       1550

GATTCAAAAT TAAAAGTTAT TAACAAATAT GTAAAAATTA TAAAAAACTA
      1560       1570       1580       1590       1600

ACCTAGTTAT AATTACTTTC CCCTCTTTTT TTTTTTTTTT TTTGTCATGA
      1610       1620       1630       1640       1650

CACTTTTTTT TTTTTGTCAT GACACTTTTT TTTTAAAAAA AAAAAAAAAA
      1660       1670       1680       1690       1700

ATGTTAAAAT ACTATTTGAT GACATTCATT TTTCCTAGTT TTTTTTTAGA
      1710       1720       1730       1740       1750
                            ClaI
TAGATATAAA AATAAATTGC CTATCGATAT ATACTTAATT TATTAAGATT
      1760       1770       1780       1790       1800
```

Fig. 1-3

```
GAATAATATT TTAATTTTTA ATAAATTCTA CTTTTTTTTT TTTTTTCTTT
      1810       1820       1830       1840       1850
                                        BglII
TTTTTTTAAA TTTTAAAATT TTTTTTTTTT ATTAGATCTC ATAATTAAAA
      1860       1870       1880       1890       1900

ATCAATTTAA AATTAAAAGT TATTTTTAAA TATGCAAAAA CTATAAAAAA
      1910       1920       1930       1940       1950

CTAATGTAGT TTAACCAACT TTTTTCTATT TCTTTTTTTT TTTTTTTTTT
      1960       1970       1980       1990       2000

TTTTTACTTT GAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AACCCTCATT
      2010       2020       2030       2040       2050

ATAAATATTA ATTACTTTGG TTTTTTTTGA TTTTTTTTTT AATAAATTTA
      2060       2070       2080       2090       2100

AAATTTTATT CTCTATCTAA TTATACCTTA TTTATAAATA TTGGAATAAT
      2110       2120       2130       2140       2150

ATATCAAATA TTTATCAGTT TTGGCATGAC AATTTTAATT ATATTTATTT
      2160       2170       2180       2190       2200

TTTGATTAGT TTTTTTTTTT TTTTTTTTTT AAAATTTCTT TTTTTTTTTT
      2210       2220       2230       2240       2250

TTTATTTTTA ATTTTTAATT TTTATTTTTC CCACACTTTC ATTTTATTTT
      2260       2270       2280       2290       2300

ATTTTATTTA TTGTAAATTC ATTTTATTTA TTTTTAATTA AATAGTTTTG
      2310       2320       2330       2340       2350
                            START
GTTTAATTTT ATTCAAAGAT TTTAAAAATG GACGAACTTA TTTCTTGGGA
      2360       2370       2380       2390       2400
                                        EcoRI
TAGGTTTTTT AAGTTTTTTG TAATACTTTT GGAAGAATTC AAAGGTTGTA
      2410       2420       2430       2440       2450

AAAGAAATGA TGTGCGTTTG AGTGTCGATT ATGACATTCT TTCTGGTATT
      2460       2470       2480       2490       2500

TATTCGCCAC GTACATTTGT ACTAAAGGAA GTCTTTAGAG CAGTGGCCGT
      2510       2520       2530       2540       2550

CTCTTATGAT GAATCTGAAA TAGATTTATT CAGATTGGGT TCAGTGTTTC
      2560       2570       2580       2590       2600

CTGGTACTTC TTTATATTCA TATATTCCAG GTATTTTCAG TTTAAAAGAT
      2610       2620       2630       2640       2650

TTCCTTTTAA TTTCAAAAAC TAAATCGGGT AAAATAAGAG TTTCGGATGT
      2660       2670       2680       2690       2700
```

Fig. 1-4

BclI

AGATCAAGCA ATATTAATTT TTGATCATTT TTCTAGAATT TCAGATAAAC
2710 2720 2730 2740 2750

AAGTATTTCG TAAAGATATT ATTCCAGGTT ATAGAACCTT TGAAAAATCA
2760 2770 2780 2790 2800

PstI

ATATCGAGCG AGTACAAAAT CTCGGATGGT CGTGCTGCAG GAGTGAGTTG
2810 2820 2830 2840 2850

GTTCAATTTA GTTAGTAAAA TAAGCACTTA TTGTAAAAAT CATCCCTTGT
2860 2870 2880 2890 2900

TTGCCGAAAA TCCAACATAT AAACATGTGG ATTTTATATC AATGTTATCA
2910 2920 2930 2940 2950

CTGGTGCATG GAATCATTGT TGATTCCCAA AATGAAGATG AGAATAATGT
2960 2970 2980 2990 3000

TTCGGCAATG TACTCTCTGA ATCCTTTTGT GGATCTTGAA AAAAGTGATA
3010 3020 3030 3040 3050

TACCAGGGGC TGTTCAAAGT AGAGTTACTA CAAATAGAAC TAGAGGTTCA
3060 3070 3080 3090 3100

AGGTCTAATT CCAATTTGAA TAATCCAACA ACAACAACAA CTACTACTAC
3110 3120 3130 3140 3150

CACTACTACA ACTACCGCAC CAATTACTAC TAGAAGTAAA AGAAAATCTG
3160 3170 3180 3190 3200

XhoI

ACGACTCTGT ACAAGAACAA AGCTCACGAC AACCAAAAAC CTCGAGAAAG
3210 3220 3230 3240 3250

TCTGGTTCTC TTAAGGATGT CAGAATTAAC AATATATCAG TAGATTCAAG
3260 3270 3280 3290 3300

TTCCAGTGAA TCTGATGTGA TTATGTCAGT TTCAAACCGT TTAAAATGTT
3310 3320 3330 3340 3350

ATCTTTTGGA AGCAGTTGTA AACAAAGGAG AGATCGGTTT AGAAGTCGTC
3360 3370 3380 3390 3400

AAAGAAGTTT TAAAAGATTT ACAGGACAAA AATTATTCCA CAGGTTTACT
3410 3420 3430 3440 3450

TGAAAACATT TTCAATCACA ACAAGTCTGA AAGGGTCATA ACACTTTCAA
3460 3470 3480 3490 3500

GTAGTTTTTT TGAAATTGCT TCAAAAATTA ACTATGATGA AGTTAAGTTC
3510 3520 3530 3540 3550

AGTGAACTCA GTATTGATGT TCTGGAATCG GCAAAGAGAT TAACATTCGA
3560 3570 3580 3590 3600

Fig. 1-5

```
GAAAAATACA AATATATTAA TTCCAACCAA TAATTTTAAA GAAGGTTTTG
      3610       3620       3630       3640       3650

AATTTTTATG GGTTCCAATT GTTAATGGTA TTGCTTCAAC TTCTGTCTTT
      3660       3670       3680       3690       3700

GTTTCACCAA ATAATTATTC AAGTGGTTCA TTTGCAAATG TAGAATCTGC
      3710       3720       3730       3740       3750

TTTAAAGTTG ATTCATCTTT GCATTTCTTT AGGAAATATA AATGGTTTCC
      3760       3770       3780       3790       3800
                                       ClaI
TCTCTATTAG ATCAATTACA TTTGATACAT TTAAATCGAT TACAAAGGAT
      3810       3820       3830       3840       3850

CTTATTCCAA TGTCGAAAAG AATGCTGGAC CTTGAACAAG GCTTCCGAAA
      3860       3870       3880       3890       3900

ACTTAGAGAT GCTTGGAATA ATAGTAATAA AAAATCCAAA GTTCAAGATA
      3910       3920       3930       3940       3950
                 ClaI                            EcoRV
GTGATATTAG TGGCATCGAT ACAGAGGATA CAAAGTTGAT ATCATTTGTC
      3960       3970       3980       3990       4000

CACGAGTTTA TAAATGATAA TTTATATTTA AAACTATCAA AAGAAGAAGA
      4010       4020       4030       4040       4050
                 AccI
TGGACTAATG CTAGTAGACT TTCCAACATC AACACTTTTT ATGAGATACA
      4060       4070       4080       4090       4100

ATCCAAATAG CATTGATAAC AAAGTTGGTT TCATGTTCCA TTGCCGTTCA
      4110       4120       4130       4140       4150

GAGATTTCAA AGTTTCAAAG TTGTAAAAAC CACTCGATAG ATAACCTTGT
      4160       4170       4180       4190       4200

TTTATCATTT ACTCCAAATA ACATTAAAAA TATATCACAG GATAATGAAA
      4210       4220       4230       4240       4250

ATGAGCTTAA AAAGAAATAT TCGTTGATGG TCAGTGATTT TAGAAATGTT
      4260       4270       4280       4290       4300

CCAAAGGTGA CACCAAAATT TATACCTTCT GAATTTAAAA GGTTTACAAT
      4310       4320       4330       4340       4350

CATTACGTTC ACAAACAATT CATACAATGC CAATAGAGTA TTTGCGTTTG
      4360       4370       4380       4390       4400

ACGACATCTC AAGTGGAATT TCAATCACAA ATGTTAAAAA TATCCACGCA
      4410       4420       4430       4440       4450
HindII
AAGGGTCAAC GAAACTTTGA AATCTACGAA ACATTACTGG GAAGTACCAG
      4460       4470       4480       4490       4500
```

Fig. 1-6

```
GATTATTCGT GCATTTTTCT GCGCTCCATG CTTGATCCAA ATCAATAATT
      4510       4520       4530       4540       4550

TTAAATTTGC CACAGATAAG TTAATTGATG ACCAAAGTGT AAATCACCAG
      4560       4570       4580       4590       4600

ATTGCATCTT TGGAAATTAA AAACTTATCA TATCTTCCGC TCGACATCAA
      4610       4620       4630       4640       4650

GGTTAGAGGT AGTACAGTTG GAACGATTAA GGGTGGAGAG ACAGCTCCTA
      4660       4670       4680       4690       4700

TTATTATAAA CTCAGAAGAA TTTACGTTTT CTATCTCATG CCTTGATATT
      4710       4720       4730       4740       4750

AGATTTAGTG CATCCTTAAT TTCTAAAACA AAACTAAGCC AACTTCCAAC
      4760       4770       4780       4790       4800

ATTTGCTCCA GATGAAAGGT ACAATAAAGA GACTAACATT TTAAAAGTTT
      4810       4820       4830       4840       4850

TGGATCAATG TGATGAACTT ACTCGAACGT TTTTAAATAA CTATAAAATA
      4860       4870       4880       4890       4900

GCTAATAAAC TATCAACCAT TGAAAATTAT TTATATAATA ATTTTATGGG
      4910       4920       4930       4940       4950

ACTAGAAGAT GAAGATGAAG ATGAAGATGA AGATGAAGAT GAAGATGAAG
      4960       4970       4980       4990       5000
                                          STOP
ATGAAGATGA AGATGAAGAT GAAGACGAAG ATGGGTATTG AATTATCATA
      5010       5020       5030       5040       5050

CTTTAAAAAT TAATTAAATA AATAAAAAAA AAAAAATGAT TTCAATTTAA
      5060       5070       5080       5090       5100

ATATATACAT ATATATATAT ATAAAATGAG ATTAATAAAA CTTTTGAGAC
      5110       5120       5130       5140       5150

CAACATTTAA TGAGATTTCT GATGCTGTTT ATTTTGCCTG GAATGAGAGC
      5160       5170       5180       5190       5200

AAAAGGCTAA AAAACATGAG AGAGAATATA ATAATAAAGG AAAACTTGGG
      5210       5220       5230       5240       5250
                                          ScaI
AAAAAGGATC TAGTATCCAT TTCCATATTA ATCCGTGCAG TACTATTAAT
      5260       5270       5280       5290       5300

TAAAAAAATA CTTTAAAAAA AATTTTAAAA ACATGGAAAA TTATATAGAT
      5310       5320       5330       5340       5350
ClaI
CGATAGATCA CTAATTTTTA AAATTAAATA TATTAAATTT ATAAAAATTG
      5360       5370       5380       5390       5400
```

Fig. 1-7

```
AAGTTCATCA AGATATATAG ATAATTATTT AATTATTTGA ATTTTTAAAA
      5410        5420        5430        5440        5450

AAAAAAAAAA AAAAAAAAAA AAAATCAAAT ATGTTTATTG TTTTAAGATT
      5460        5470        5480        5490        5500
                                          ClaI
TTTTAATCTC GTCAATGATT TTAAAATAAA AATCGATACA TAATTTTAAA
      5510        5520        5530        5540        5550

AAAAACCCTT TACATTTTTT ATTTTAATTC CAAATTTATA CATTTTTTAT
      5560        5570        5580        5590        5600

TTTTTTTTTT TTTTTTTTTT TTTTTTTTAA TTTAAATTTT TTTTTTTTTT
      5610        5620        5630        5640        5650

TTTTTTTTAT TTATTTAAAA TTTAATTATT AATTTTATAA ATAAAAAATA
      5660        5670        5680        5690        5700

GAAATATAAG TAAAAAAACA AACAACAAAT AACATATATA AAAAAATACA
      5710        5720        5730        5740        5750

AATAACAAAT AATTAAATAA ATTAAATAAC CATTAAAAAT GTATATTAAT
      5760        5770        5780        5790        5800
            BglII               ScaI
AAATTTAAAA GATCTTTATT AGTACTATTG TTACTTTGTA ATATTCTTCC
      5810        5820        5830        5840        5850
SalI
TG
```

Fig. 2-1

REP GENE:

```
          2386       2395       2404       2413       2422
ATG GAC GAA CTT ATT TCT TGG GAT AGG TTT TTT AAG TTT TTT GTA
m   d   e   l   i   s   w   d   r   f   f   k   f   f   v

          2431       2440       2449       2458       2467
ATA CTT TTG GAA GAA TTC AAA GGT TGT AAA AGA AAT GAT GTG CGT
i   l   l   e   e   f   k   g   c   k   r   n   d   v   r

          2476       2485       2494       2503       2512
TTG AGT GTC GAT TAT GAC ATT CTT TCT GGT ATT TAT TCG CCA CGT
l   s   v   d   y   d   i   l   s   g   i   y   s   p   r

          2521       2530       2539       2548       2557
ACA TTT GTA CTA AAG GAA GTC TTT AGA GCA GTG GCC GTC TCT TAT
t   f   v   l   k   e   v   f   r   a   v   a   v   s   y

          2566       2575       2584       2593       2602
GAT GAA TCT GAA ATA GAT TTA TTC AGA TTG GGT TCA GTG TTT CCT
d   e   s   e   i   d   l   f   r   l   g   s   v   f   p

          2611       2620       2629       2638       2647
GGT ACT TCT TTA TAT TCA TAT ATT CCA GGT ATT TTC AGT TTA AAA
g   t   s   l   y   s   y   i   p   g   i   f   s   l   k

          2656       2665       2674       2683       2692
GAT TTC CTT TTA ATT TCA AAA ACT AAA TCG GGT AAA ATA AGA GTT
d   f   l   l   i   s   k   t   k   s   g   k   i   r   v

          2701       2710       2719       2728       2737
TCG GAT GTA GAT CAA GCA ATA TTA ATT TTT GAT CAT TTT TCT AGA
s   d   v   d   q   a   i   l   i   f   d   h   f   s   r

          2746       2755       2764       2773       2782
ATT TCA GAT AAA CAA GTA TTT CGT AAA GAT ATT ATT CCA GGT TAT
i   s   d   k   q   v   f   r   k   d   i   i   p   g   y

          2791       2800       2809       2818       2827
AGA ACC TTT GAA AAA TCA ATA TCG AGC GAG TAC AAA ATC TCG GAT
r   t   f   e   k   s   i   s   s   e   y   k   i   s   d

          2836       2845       2854       2863       2872
GGT CGT GCT GCA GGA GTG AGT TGG TTC AAT TTA GTT AGT AAA ATA
g   r   a   a   g   v   s   w   f   n   l   v   s   k   i

          2881       2890       2899       2908       2917
AGC ACT TAT TGT AAA AAT CAT CCC TTG TTT GCC GAA AAT CCA ACA
s   t   y   c   k   n   h   p   l   f   a   e   n   p   t

          2926       2935       2944       2953       2962
TAT AAA CAT GTG GAT TTT ATA TCA ATG TTA TCA CTG GTG CAT GGA
y   k   h   v   d   f   i   s   m   l   s   l   v   h   g
```

Fig. 2-2

```
      2971        2980        2989        2998        3007
ATC ATT GTT GAT TCC CAA AAT GAA GAT GAG AAT AAT GTT TCG GCA
i   i   v   d   s   q   n   e   d   e   n   n   v   s   a

      3016        3025        3034        3043        3052
ATG TAC TCT CTG AAT CCT TTT GTG GAT CTT GAA AAA AGT GAT ATA
m   y   s   l   n   p   f   v   d   l   e   k   s   d   i

      3061        3070        3079        3088        3097
CCA GGG GCT GTT CAA AGT AGA GTT ACT ACA AAT AGA ACT AGA GGT
p   g   a   v   q   s   r   v   t   t   n   r   t   r   g

      3106        3115        3124        3133        3142
TCA AGG TCT AAT TCC AAT TTG AAT AAT CCA ACA ACA ACA ACA ACT
s   r   s   n   s   n   l   n   n   p   t   t   t   t   t

      3151        3160        3169        3178        3187
ACT ACT ACC ACT ACT ACA ACT ACC GCA CCA ATT ACT ACT AGA AGT
t   t   t   t   t   t   t   t   a   p   i   t   t   r   s

      3196        3205        3214        3223        3232
AAA AGA AAA TCT GAC GAC TCT GTA CAA GAA CAA AGC TCA CGA CAA
k   r   k   s   d   d   s   v   q   e   q   s   s   r   q

      3241        3250        3259        3268        3277
CCA AAA ACC TCG AGA AAG TCT GGT TCT CTT AAG GAT GTC AGA ATT
p   k   t   s   r   k   s   g   s   l   k   d   v   r   i

      3286        3295        3304        3313        3322
AAC AAT ATA TCA GTA GAT TCA AGT TCC AGT GAA TCT GAT GTG ATT
n   n   i   s   v   d   s   s   s   s   e   s   d   v   i

      3331        3340        3349        3358        3367
ATG TCA GTT TCA AAC CGT TTA AAA TGT TAT CTT TTG GAA GCA GTT
m   s   v   s   n   r   l   k   c   y   l   l   e   a   v

      3376        3385        3394        3403        3412
GTA AAC AAA GGA GAG ATC GGT TTA GAA GTC GTC AAA GAA GTT TTA
v   n   k   g   e   i   g   l   e   v   v   k   e   v   l

      3421        3430        3439        3448        3457
AAA GAT TTA CAG GAC AAA AAT TAT TCC ACA GGT TTA CTT GAA AAC
k   d   l   q   d   k   n   y   s   t   g   l   l   e   n

      3466        3475        3484        3493        3502
ATT TTC AAT CAC AAC AAG TCT GAA AGG GTC ATA ACA CTT TCA AGT
i   f   n   h   n   k   s   e   r   v   i   t   l   s   s

      3511        3520        3529        3538        3547
AGT TTT TTT GAA ATT GCT TCA AAA ATT AAC TAT GAT GAA GTT AAG
s   f   f   e   i   a   s   k   i   n   y   d   e   v   k

      3556        3565        3574        3583        3592
TTC AGT GAA CTC AGT ATT GAT GTT CTG GAA TCG GCA AAG AGA TTA
f   s   e   l   s   i   d   v   l   e   s   a   k   r   l
```

Fig. 2-3

```
      3601        3610        3619        3628        3637
ACA TTC GAG AAA AAT ACA AAT ATA TTA ATT CCA ACC AAT AAT TTT
t   f   e   k   n   t   n   i   l   i   p   t   n   n   f

      3646        3655        3664        3673        3682
AAA GAA GGT TTT GAA TTT TTA TGG GTT CCA ATT GTT AAT GGT ATT
k   e   g   f   e   f   l   w   v   p   i   v   n   g   i

      3691        3700        3709        3718        3727
GCT TCA ACT TCT GTC TTT GTT TCA CCA AAT AAT TAT TCA AGT GGT
a   s   t   s   v   f   v   s   p   n   n   y   s   s   g

      3736        3745        3754        3763        3772
TCA TTT GCA AAT GTA GAA TCT GCT TTA AAG TTG ATT CAT CTT TGC
s   f   a   n   v   e   s   a   l   k   l   i   h   l   c

      3781        3790        3799        3808        3817
ATT TCT TTA GGA AAT ATA AAT GGT TTC CTC TCT ATT AGA TCA ATT
i   s   l   g   n   i   n   g   f   l   s   i   r   s   i

      3826        3835        3844        3853        3862
ACA TTT GAT ACA TTT AAA TCG ATT ACA AAG GAT CTT ATT CCA ATG
t   f   d   t   f   k   s   i   t   k   d   l   i   p   m

      3871        3880        3889        3898        3907
TCG AAA AGA ATG CTG GAC CTT GAA CAA GGC TTC CGA AAA CTT AGA
s   k   r   m   l   d   l   e   q   g   f   r   k   l   r

      3916        3925        3934        3943        3952
GAT GCT TGG AAT AAT AGT AAT AAA AAA TCC AAA GTT CAA GAT AGT
d   a   w   n   n   s   n   k   k   s   k   v   q   d   s

      3961        3970        3979        3988        3997
GAT ATT AGT GGC ATC GAT ACA GAG GAT ACA AAG TTG ATA TCA TTT
d   i   s   g   i   d   t   e   d   t   k   l   i   s   f

      4006        4015        4024        4033        4042
GTC CAC GAG TTT ATA AAT GAT AAT TTA TAT TTA AAA CTA TCA AAA
v   h   e   f   i   n   d   n   l   y   l   k   l   s   k

      4051        4060        4069        4078        4087
GAA GAA GAT GGA CTA ATG CTA GTA GAC TTT CCA ACA TCA ACA CTT
e   e   d   g   l   m   l   v   d   f   p   t   s   t   l

      4096        4105        4114        4123        4132
TTT ATG AGA TAC AAT CCA AAT AGC ATT GAT AAC AAA GTT GGT TTC
f   m   r   y   n   p   n   s   i   d   n   k   v   g   f

      4141        4150        4159        4168        4177
ATG TTC CAT TGC CGT TCA GAG ATT TCA AAG TTT CAA AGT TGT AAA
m   f   h   c   r   s   e   i   s   k   f   q   s   c   k

      4186        4195        4204        4213        4222
AAC CAC TCG ATA GAT AAC CTT GTT TTA TCA TTT ACT CCA AAT AAC
n   h   s   i   d   n   l   v   l   s   f   t   p   n   n
```

Fig. 2-4

```
      4231        4240        4249        4258        4267
ATT AAA AAT ATA TCA CAG GAT AAT GAA AAT GAG CTT AAA AAG AAA
i   k   n   i   s   q   d   n   e   n   e   l   k   k   k

      4276        4285        4294        4303        4312
TAT TCG TTG ATG GTC AGT GAT TTT AGA AAT GTT CCA AAG GTG ACA
y   s   l   m   v   s   d   f   r   n   v   p   k   v   t

      4321        4330        4339        4348        4357
CCA AAA TTT ATA CCT TCT GAA TTT AAA AGG TTT ACA ATC ATT ACG
p   k   f   i   p   s   e   f   k   r   f   t   i   i   t

      4366        4375        4384        4393        4402
TTC ACA AAC AAT TCA TAC AAT GCC AAT AGA GTA TTT GCG TTT GAC
f   t   n   n   s   y   n   a   n   r   v   f   a   f   d

      4411        4420        4429        4438        4447
GAC ATC TCA AGT GGA ATT TCA ATC ACA AAT GTT AAA AAT ATC CAC
d   i   s   s   g   i   s   i   t   n   v   k   n   i   h

      4456        4465        4474        4483        4492
GCA AAG GGT CAA CGA AAC TTT GAA ATC TAC GAA ACA TTA CTG GGA
a   k   g   q   r   n   f   e   i   y   e   t   l   l   g

      4501        4510        4519        4528        4537
AGT ACC AGG ATT ATT CGT GCA TTT TTC TGC GCT CCA TGC TTG ATC
s   t   r   i   i   r   a   f   f   c   a   p   c   l   i

      4546        4555        4564        4573        4582
CAA ATC AAT AAT TTT AAA TTT GCC ACA GAT AAG TTA ATT GAT GAC
q   i   n   n   f   k   f   a   t   d   k   l   i   d   d

      4591        4600        4609        4618        4627
CAA AGT GTA AAT CAC CAG ATT GCA TCT TTG GAA ATT AAA AAC TTA
q   s   v   n   h   q   i   a   s   l   e   i   k   n   l

      4636        4645        4654        4663        4672
TCA TAT CTT CCG CTC GAC ATC AAG GTT AGA GGT AGT ACA GTT GGA
s   y   l   p   l   d   i   k   v   r   g   s   t   v   g

      4681        4690        4699        4708        4717
ACG ATT AAG GGT GGA GAG ACA GCT CCT ATT ATT ATA AAC TCA GAA
t   i   k   g   g   e   t   a   p   i   i   i   n   s   e

      4726        4735        4744        4753        4762
GAA TTT ACG TTT TCT ATC TCA TGC CTT GAT ATT AGA TTT AGT GCA
e   f   t   f   s   i   s   c   l   d   i   r   f   s   a

      4771        4780        4789        4798        4807
TCC TTA ATT TCT AAA ACA AAA CTA AGC CAA CTT CCA ACA TTT GCT
s   l   i   s   k   t   k   l   s   q   l   p   t   f   a

      4816        4825        4834        4843        4852
CCA GAT GAA AGG TAC AAT AAA GAG ACT AAC ATT TTA AAA GTT TTG
p   d   e   r   y   n   k   e   t   n   i   l   k   v   l
```

Fig. 2-5

```
        4861                4870                4879                4888                4897
GAT CAA TGT GAT GAA CTT ACT CGA ACG TTT TTA AAT AAC TAT AAA
d   q   c   d   e   l   t   r   t   f   l   n   n   y   k

        4906                4915                4924                4933                4942
ATA GCT AAT AAA CTA TCA ACC ATT GAA AAT TAT TTA TAT AAT AAT
i   a   n   k   l   s   t   i   e   n   y   l   y   n   n

        4951                4960                4969                4978                4987
TTT ATG GGA CTA GAA GAT GAA GAT GAA GAT GAA GAT GAA GAT GAA
f   m   g   l   e   d   e   d   e   d   e   d   e   d   e

        4996                5005                5014                5023                5032
GAT GAA GAT GAA GAT GAA GAT GAA GAT GAA GAT GAA GAC GAA GAT
d   e   d   e   d   e   d   e   d   e   d   e   d   e   d


GGG TAT
g   y
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Fig. 3

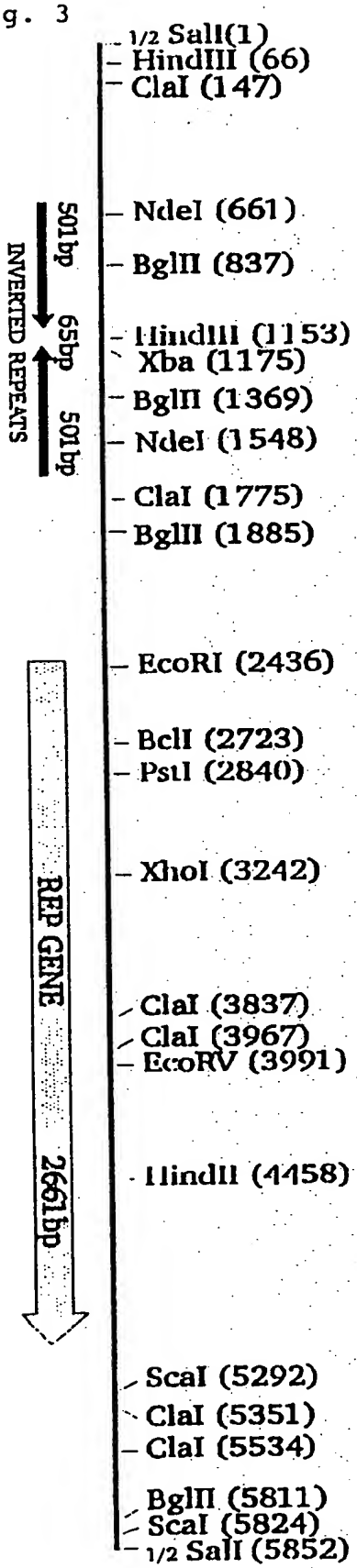
1/2 SalI(1)
HindIII (66)
ClaI (147)
NdeI (661)
BglII (837)
HindIII (1153)
Xba (1175)
BglII (1369)
NdeI (1548)
ClaI (1775)
BglII (1885)
EcoRI (2436)
BclI (2723)
PstI (2840)
XhoI (3242)
ClaI (3837)
ClaI (3967)
EcoRV (3991)
HindII (4458)
ScaI (5292)
ClaI (5351)
ClaI (5534)
BglII (5811)
ScaI (5824)
1/2 SalI (5852)
501bp
65bp
501bp
INVERTED REPEATS
REP GENE
2661bp

Fig. 4

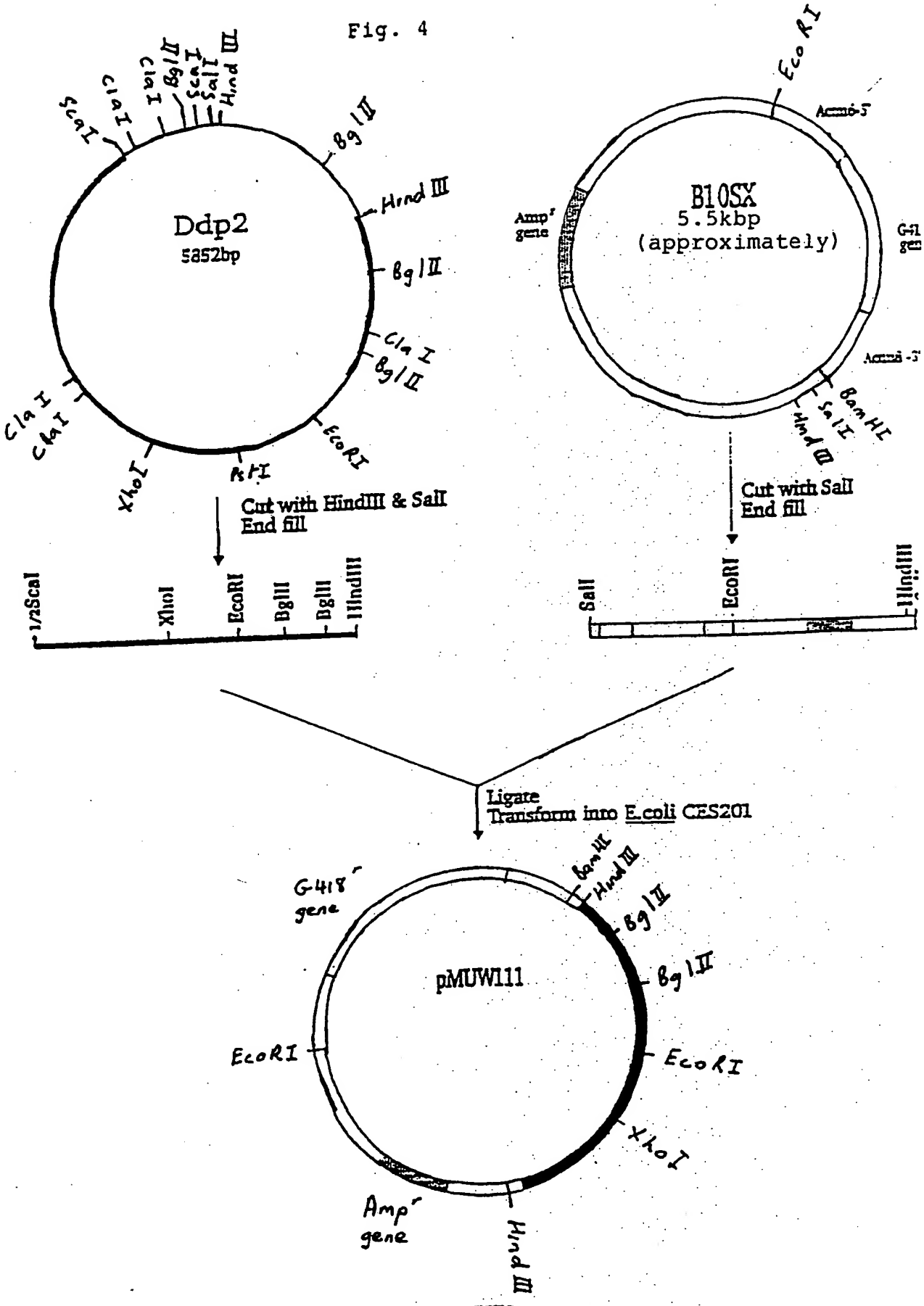

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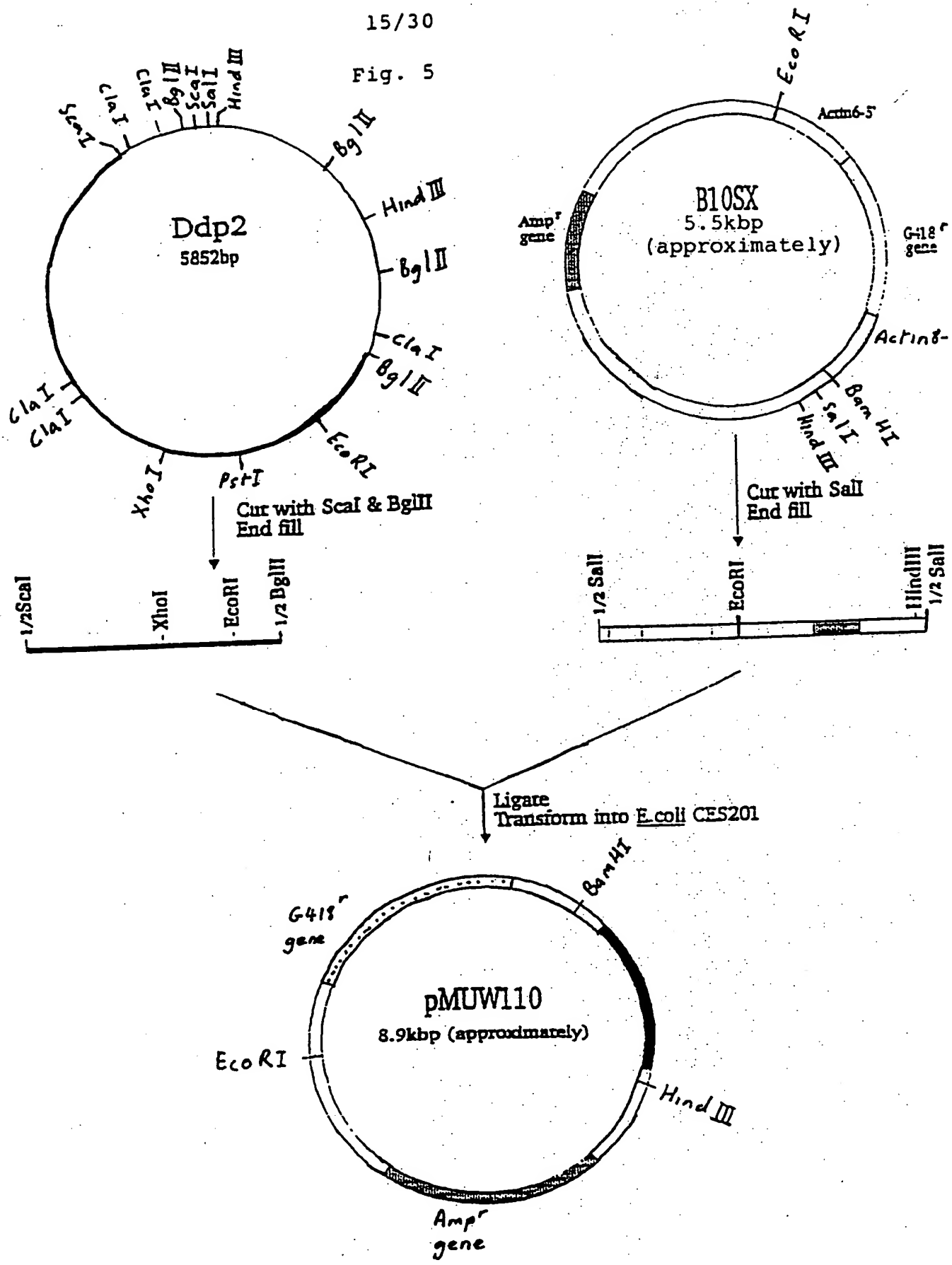
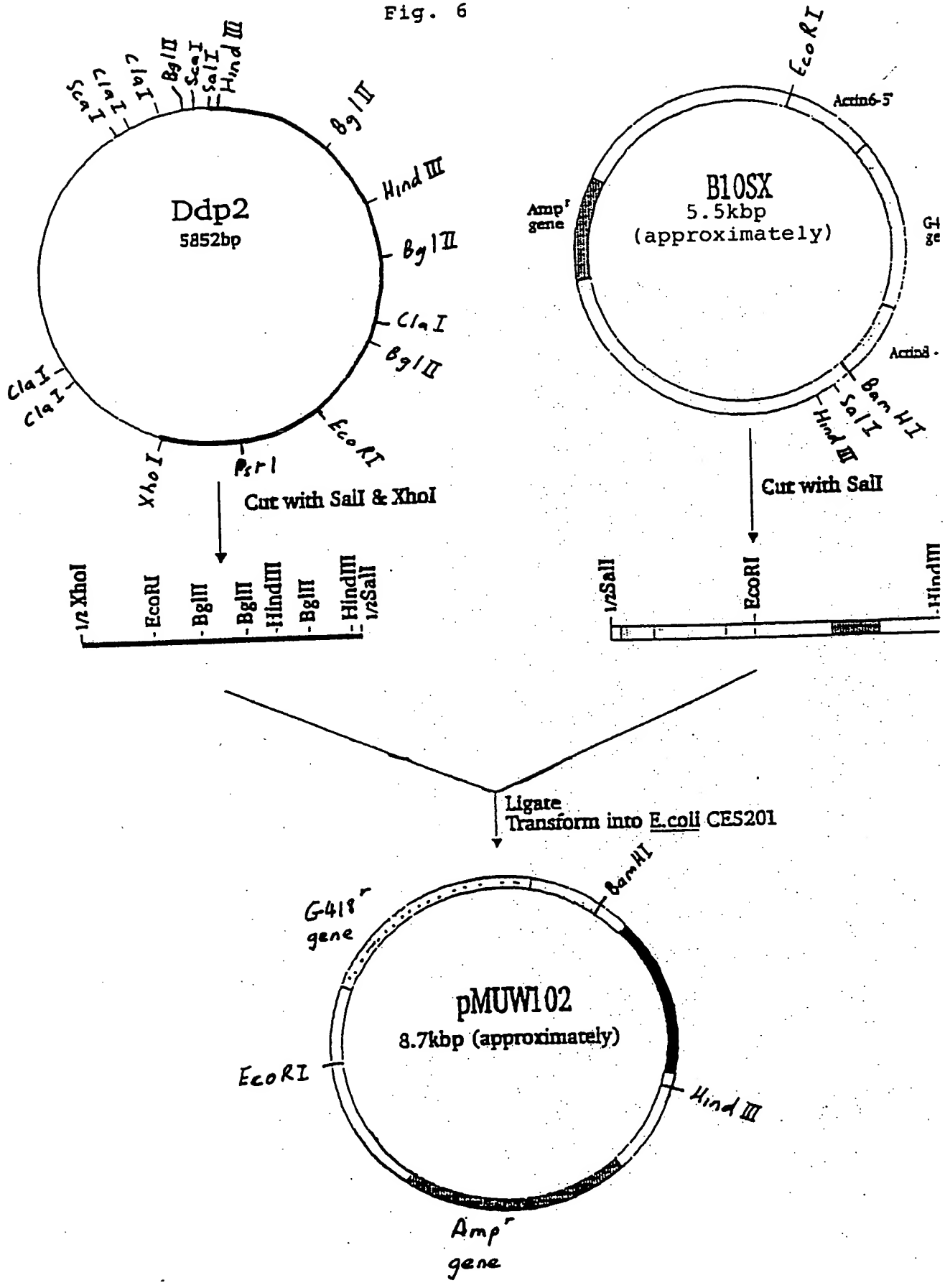
Fig. 5



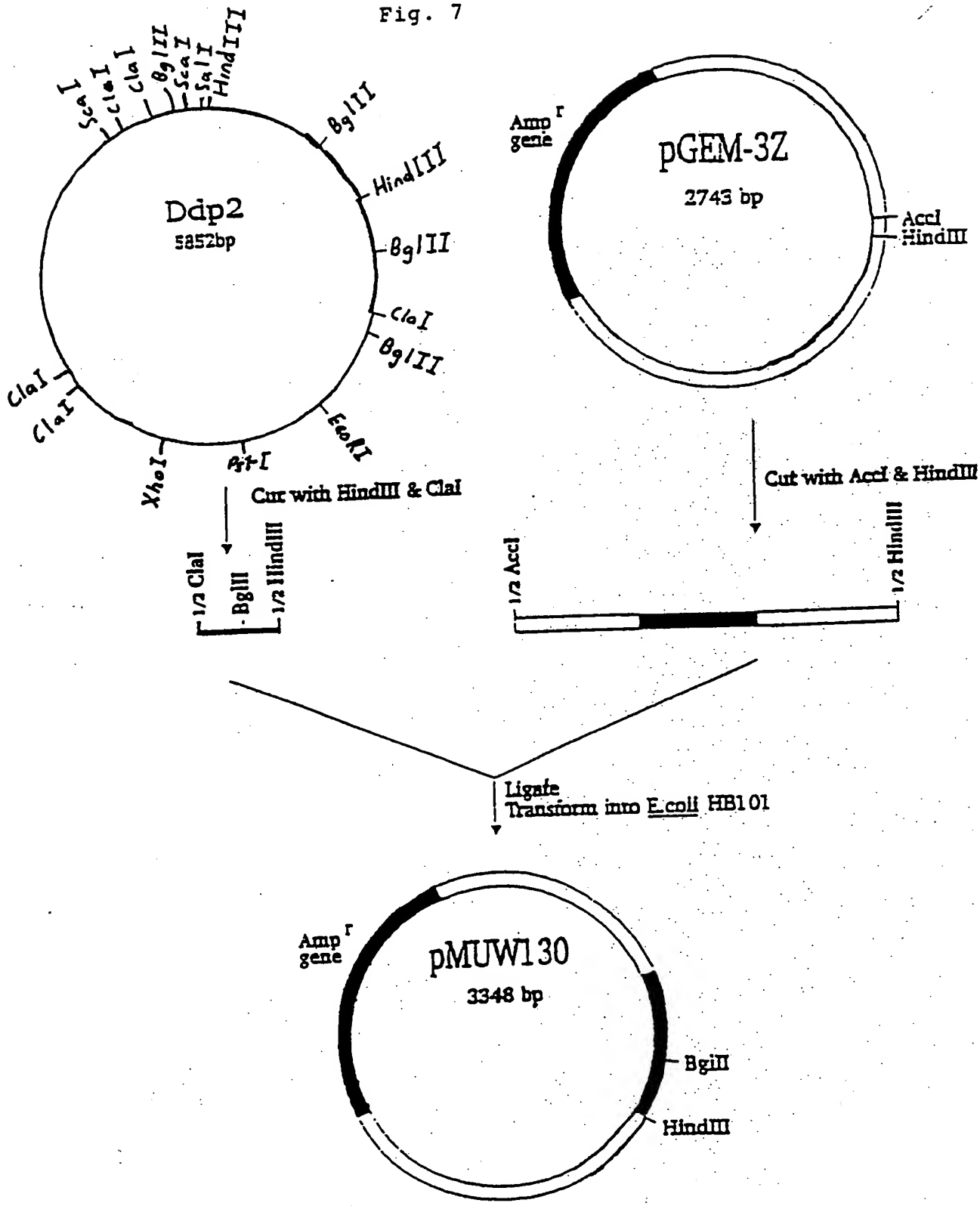
SUBSTITUTE SHEET

Fig. 6



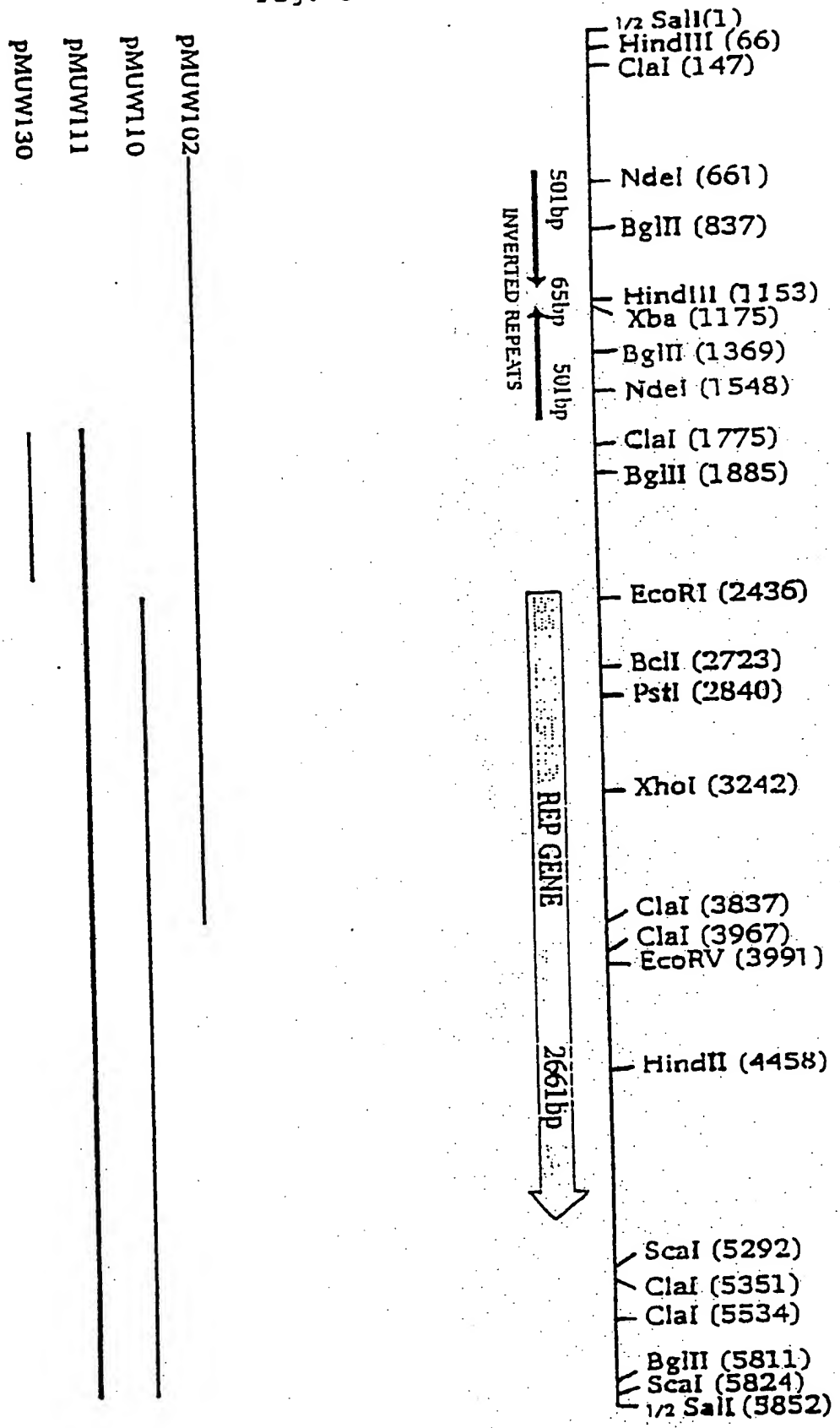
SUBSTITUTE SHEET

Fig. 7



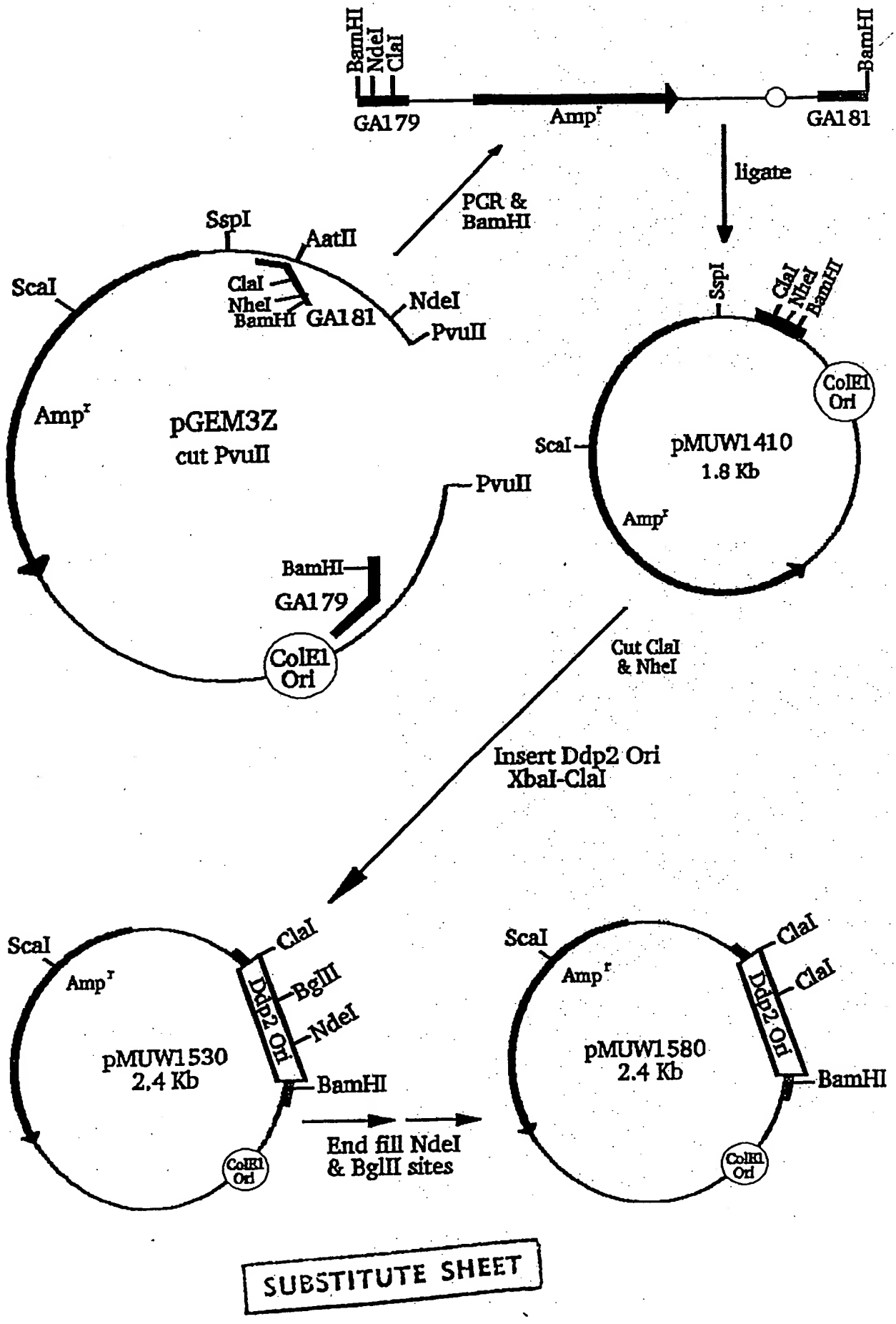
SUBSTITUTE SHEET

Fig. 8



SUBSTITUTE SHEET

Fig. 9



SUBSTITUTE SHEET

Fig. 10-1

ClaI
CGATAGGTGG CACTTTTCGG GGAAATGTGC GCGGAACCCC TATTTGTTTA

TTTTTCTAAA TACATTCAAA TATGTATCCG CTCATGAGAC AATAACCCTG

ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT ATTCAACATT

TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT TCCTGTTTTT

GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG

TGCACGAGTG GGTTACATCG AACTGGATCT CAACAGCGGT AAGATCCTTG

AGAGTTTTCG CCCCGAAGAA CGTTTTCCAA TGATGAGCAC TTTTAAAGTT

CTGCTATGTG GCGCGGTATT ATCCCGTATT GACGCCGGGC AAGAGCAACT

ScaI
CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG TACTCACCAG

TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT

GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC

GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC

ATGTAACTCG CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA

AACGACGAGC GTGACACCAC GATGCCTGTA GCAATGCCAA CAACGTTGCG

CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG CAACAATTAA

TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC

CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG

GTCTCGCGGT ATCATTGCAG CACTGGGGCC AGATGGTAAG CCCTCCCGTA

Fig. 10-2

```
      910         920         930         940         950
TCGTAGTTAT CTACACGACG GGGAGTCAGG CAACTATGGA TGAACGAAAT

      960         970         980         990        1000
AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT GGTAACTGTC

     1010        1020        1030        1040        1050
AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA CTTCATTTTT

     1060        1070        1080        1090        1100
AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA

     1110        1120        1130        1140        1150
ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA

     1160        1170        1180        1190        1200
GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT

     1210        1220        1230        1240        1250
TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA

     1260        1270        1280        1290        1300
GAGCTACCAA CTCTTTTTCC GAAGGTAACT GGCTTCAGCA GAGCGCAGAT

     1310        1320        1330        1340        1350
ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA

     1360        1370        1380        1390        1400
ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG

     1410        1420        1430        1440        1450
GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG

     1460        1470        1480        1490        1500
ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA

     1510        1520        1530        1540        1550
CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG

     1560        1570        1580        1590        1600
CGTGAGCTAT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA AGGCGGACAG

     1610        1620        1630        1640        1650
GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC

     1660        1670        1680        1690        1700
CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC

     1710        1720        1730        1740        1750
TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATC

     1760        1770        1780        1790        1800
GAAAAACGCC AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC
```

Fig. 10-3

```
      1810        BamHI       1830        1840        1850
CTTTTGCTGG CCTTTGGATC CGCTAGACGA GCACAAATAT ATACTTTTTA

      1860        1870        1880        1890        1900
TTAAAAACGG AGGTCATTTC AATACCTATT GAAGAAATAA ATTTTTTTTT

      1910        1920        1930        1940        1950
TTTTTTTTTT TGTCATGACA CTTTTTTTTT TTTGTCATGA CAGAATTGAA

      1960        1970        1980        1990        2000
AAAAACAGAA AGTTATATAT TTACCCCCTT TAATTTTTTT TAAAACTTTT

      2010        BglII 2030        2040        2050
GAAACTTTAG TAATAAGATC TATACTTCAG TACGAACATA AATATGTATA

      2060        2070        2080        2090        2100
AACCAAAAAA ATTGATTAAG ATAAAGTTAT ATGTTTGTAT TTAATAAAAT

      2110        2120        2130        2140        2150
AGTTTAGTTT AAAATTTTAT ATCATTTTTT AAAAAATGAA AATGTTTGAA

      2160        2170        2180        2190   NdeI2200
AAAAAAAATT TTTTTTTTTT TTTTCAACGG GACGATGTAA TATCATATGA

      2210        2220        2230        2240        2250
TTCAAAATTA AAAGTTATTA ACAAATATGT AAAAATTATA AAAAACTAAC

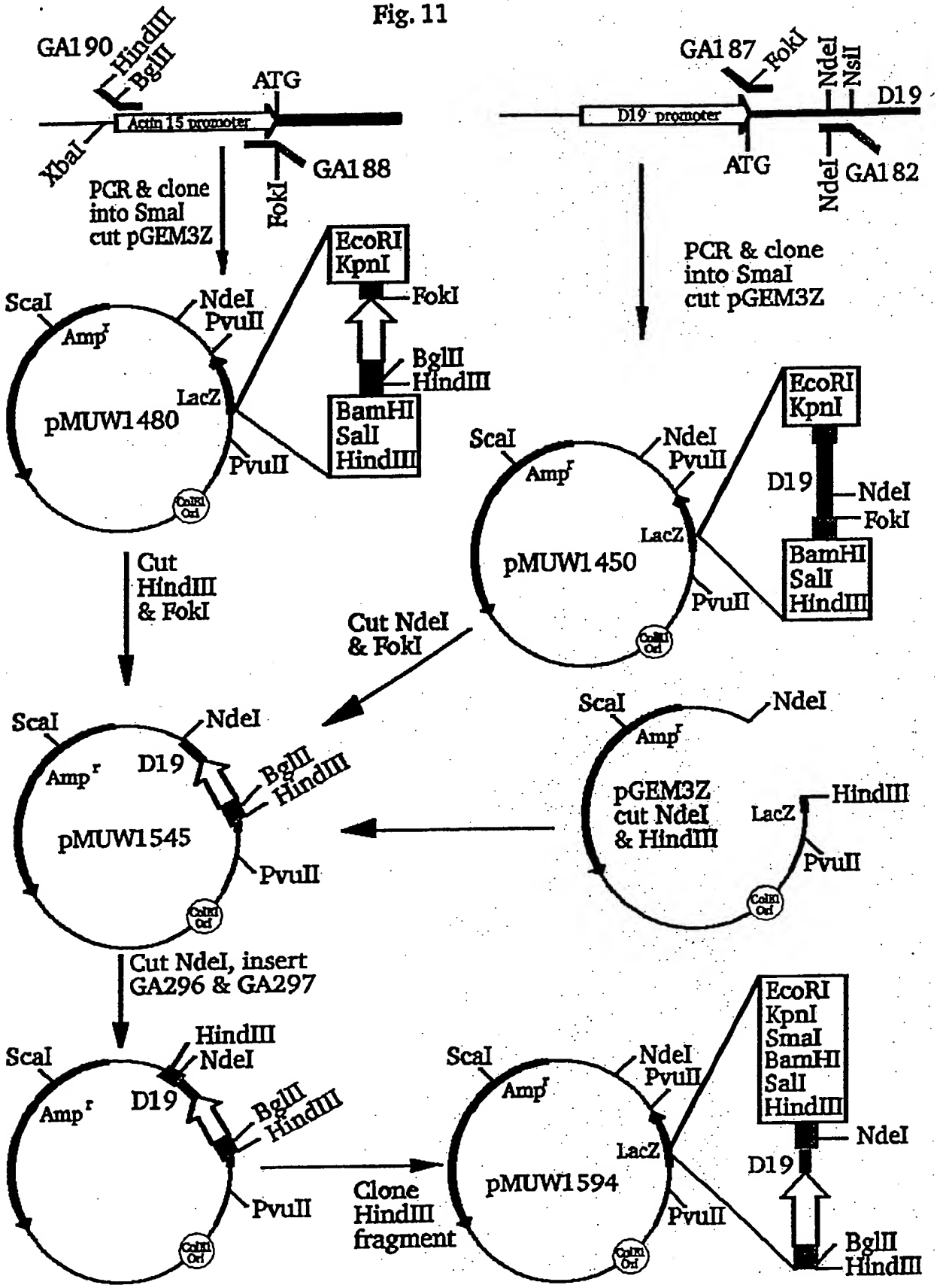
      2260        2270        2280        2290        2300
CTAGTTATAA TTACTTTCCC CTCTTTTTTT TTTTTTTTTT TGTCATGACA

      2310        2320        2330        2340        2350
CTTTTTTTTT TTTGTCATGA CACTTTTTTT TTAAAAAAAA AAAAAAAAAT

      2360        2370        2380        2390        2400
GTTAAAATAC TATTTGATGA CATTCATTTT TCCTAGTTTT TTTTTAGATA

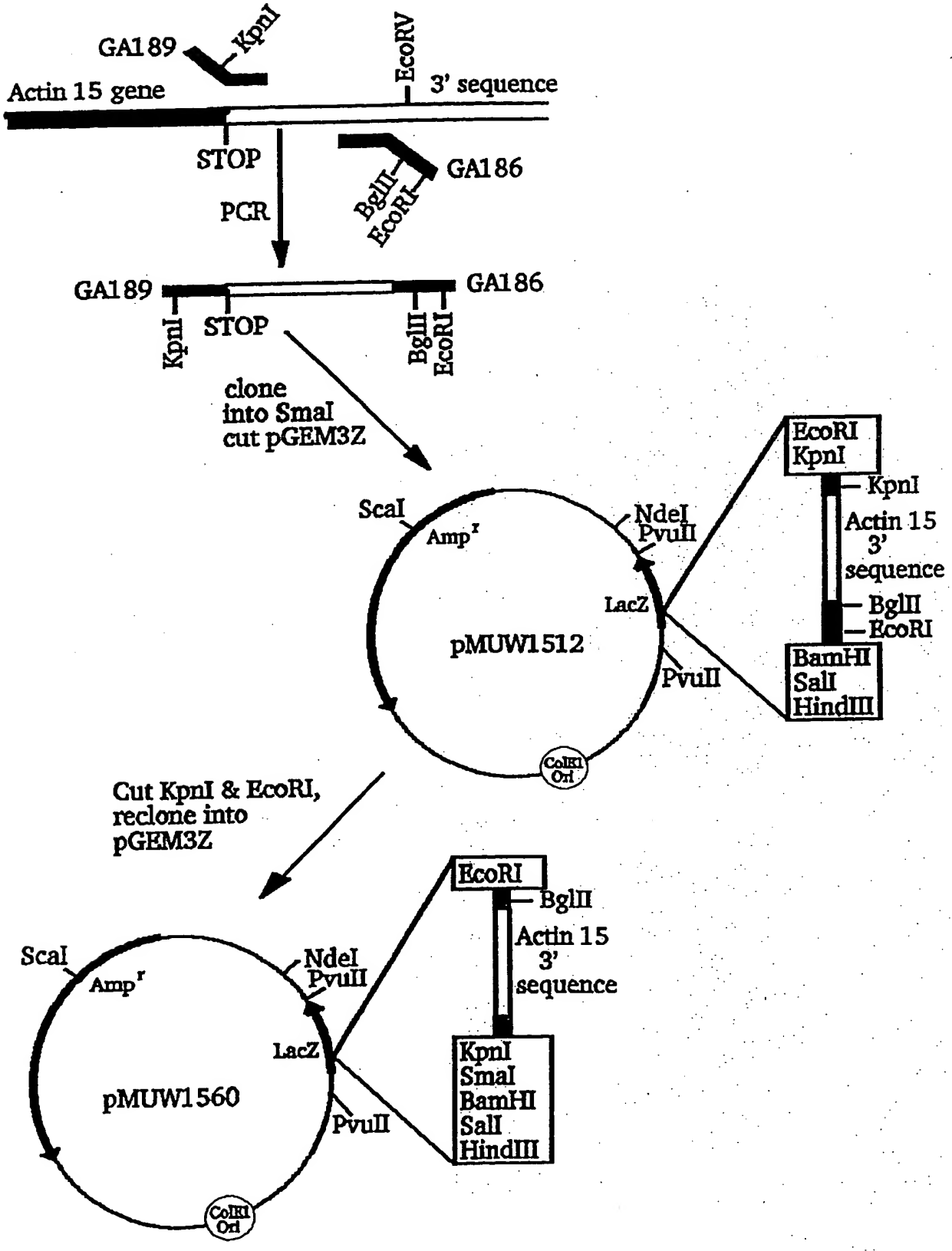
      2410        2420 ClaI
GATATAAAAA TAAATTGCCT AT
```

Fig. 11



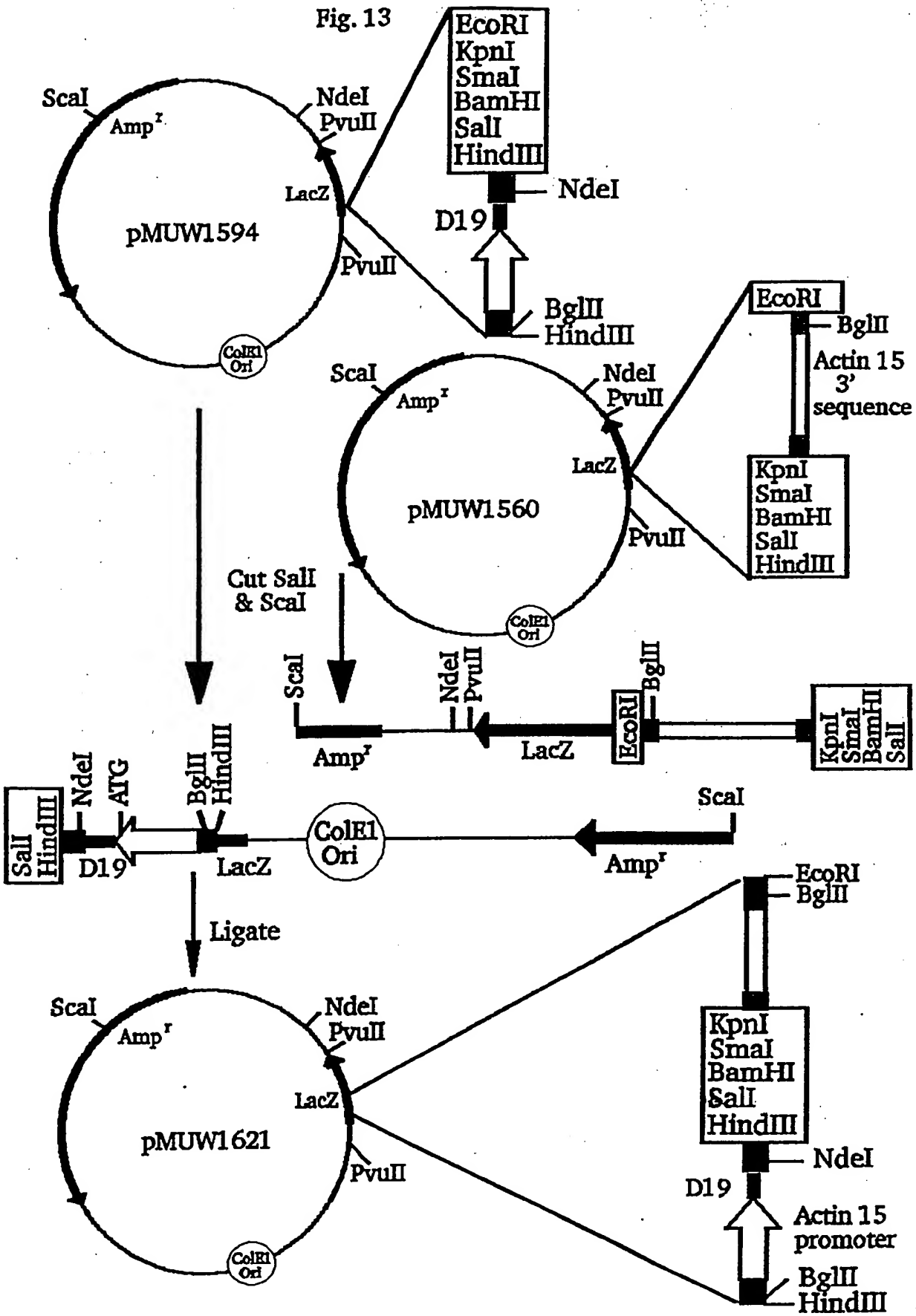
SUBSTITUTE SHEET

Fig. 12



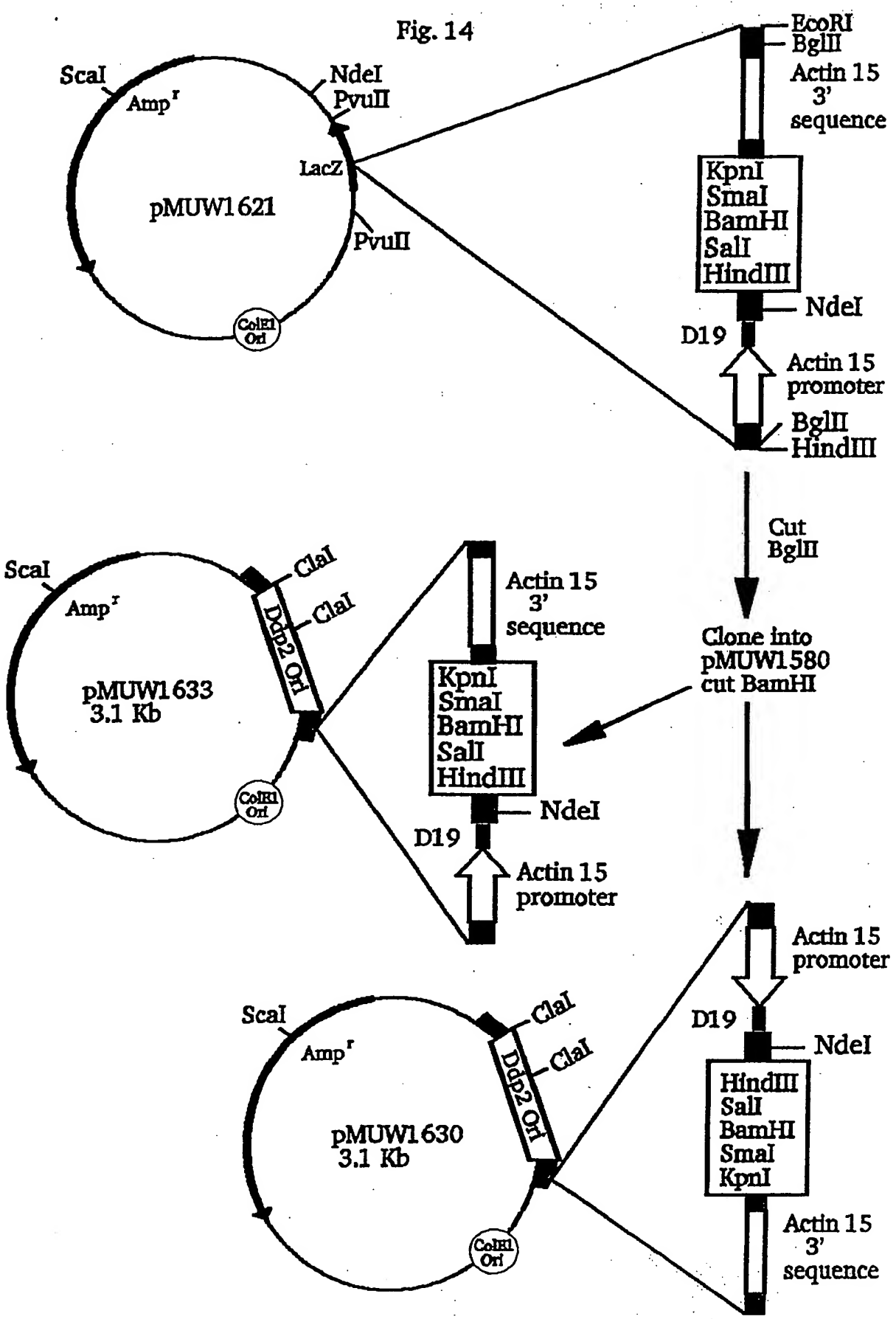
SUBSTITUTE SHEET

Fig. 13



SUBSTITUTE SHEET

Fig. 14


ScaI
Amp r
NdeI
PvuII
LacZ
pMUW1621
PvuII
ColE1 Ori
EcoRI
BglII
Actin 15
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sequence
KpnI
SmaI
BamHI
SalI
HindIII
NdeI
D19
Actin 15
promoter
BglII
HindIII
Cut
BglII
ScaI
Amp r
ClaI
ClaI
Ddp2 Ori
pMUW1633
3.1 Kb
ColE1 Ori
Actin 15
3'
sequence
KpnI
SmaI
BamHI
SalI
HindIII
NdeI
D19
Actin 15
promoter
Clone into
pMUW1580
cut BamHI
Actin 15
promoter
D19
NdeI
HindIII
SalI
BamHI
SmaI
KpnI
Actin 15
3'
sequence
ScaI
Amp r
ClaI
ClaI
Ddp2 Ori
pMUW1630
3.1 Kb
ColE1 Ori

Fig. 15-1

ClaI
```
        10         20         30         40         50
CGATAGGTGG CACTTTTCGG GGAAATGTGC GCGGAACCCC TATTTGTTTA

        60         70         80         90        100
TTTTTCTAAA TACATTCAAA TATGTATCCG CTCATGAGAC AATAACCCTG

       110        120        130        140        150
ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT ATTCAACATT

       160        170        180        190        200
TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT TCCTGTTTTT

       210        220        230        240        250
GCTCACCCAG AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG

       260        270        280        290        300
TGCACGAGTG GGTTACATCG AACTGGATCT CAACAGCGGT AAGATCCTTG

       310        320        330        340        350
AGAGTTTTCG CCCCGAAGAA CGTTTTCCAA TGATGAGCAC TTTTAAAGTT

       360        370        380        390        400
CTGCTATGTG GCGCGGTATT ATCCCGTATT GACGCCGGGC AAGAGCAACT

       410        420        430        440 ScaI   450
CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG TACTCACCAG

       460        470        480        490        500
TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT

       510        520        530        540        550
GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC

       560        570        580        590        600
GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC

       610        620        630        640        650
ATGTAACTCG CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA

       660        670        680        690        700
AACGACGAGC GTGACACCAC GATGCCTGTA GCAATGCCAA CAACGTTGCG

       710        720        730        740        750
CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG CAACAATTAA

       760        770        780        790        800
TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC

       810        820        830        840        850
CTTCCGGCTG GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG

       860        870        880        890        900
GTCTCGCGGT ATCATTGCAG CACTGGGGCC AGATGGTAAG CCCTCCCGTA
```

Fig. 15-2

```
      910         920         930         940         950
TCGTAGTTAT CTACACGACG GGGAGTCAGG CAACTATGGA TGAACGAAAT

      960         970         980         990        1000
AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT GGTAACTGTC

     1010        1020        1030        1040        1050
AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA CTTCATTTTT

     1060        1070        1080        1090        1100
AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA

     1110        1120        1130        1140        1150
ATCCCTTAAC GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA

     1160        1170        1180        1190        1200
GATCAAAGGA TCTTCTTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT

     1210        1220        1230        1240        1250
TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA

     1260        1270        1280        1290        1300
GAGCTACCAA CTCTTTTTCC GAAGGTAACT GGCTTCAGCA GAGCGCAGAT

     1310        1320        1330        1340        1350
ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA

     1360        1370        1380        1390        1400
ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG

     1410        1420        1430        1440        1450
GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG

     1460        1470        1480        1490        1500
ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA

     1510        1520        1530        1540        1550
CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG

     1560        1570        1580        1590        1600
CGTGAGCTAT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA AGGCGGACAG

     1610        1620        1630        1640        1650
GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC

     1660        1670        1680        1690        1700
CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC

     1710        1720        1730        1740        1750
TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATC

     1760        1770        1780        1790        1800
GAAAAACGCC AGCAACGCGG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC
```

Fig. 15-3

```
      1810        1820        1830        1840        1850
CTTTTGCTGG CCTTTGGATC TACAAATTAA TTAATCCCAT CAAATCTTTA

      1860        1870        1880        1890        1900
AAAAAAAAAA TGGTTTAAAA AAACTTGGGT TGGTTAATTA TTATTTGAAA

      1910        1920        1930        1940        1950
ATTTTAAAAC CCAAATTAAA AAAAAAAAAT GGGATTCAAA AATTTTTTTT

      1960        1970        1980        1990        2000
TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTCA GATTGCATAA

      2010        2020        2030        2040        2050
AAAGATTTTT TTTTTTTTTT TTTCTTATTT CTTAAAACAA ATAAATTAAA

      2060         START      2080        2090        2100
TTAAATAAAA AATAAAAATG AAATTCCAAC ATACATTTAT TGCATTATTA

      2110        2120 NsiI    HindIII 2140        2150
TCACTATTAA CATACGCCAA TGCATATGAA AGCTTGCATG CCTGCAGGTC

      2160       SmaI KpnI    2180        2190        2200
GACTCTAGAG GATCCCCGGG TACCTAAATC ATGAATGAAA GTGCTTCACA

      2210        2220        2230        2240        2250
TAAAAATAAT AATAATAATA TAACAATAAT AATATTTAAA TGTATAATAA

      2260        2270        2280        2290        2300
AATTTAATTA CTTTTTTTTT AATGGTTGTT GATCTTTATC CGACCTTAAA

      2310        2320        2330        2340        2350
AAAAAAAAAA TAAAACCAAT AGGCTATTGG TTTTTTTTTT AATTGTTTTT

      2360        2370        2380        2390        2400
TTATTTTTTA TTATTACTTT AATTATCATT TTTTAAATTA CAAAAAAAAT

      2410        2420        2430        2440        2450
TAAAAATCCA GATATTAAGG TATTTGCACT AGTGCTTTAA CGTTAAAATT

      2460        2470        2480        2490        2500
TGAAAAAAAA AAAAAATTAA TAATTTTACC CTTTATGGGT AAACGATTCT

      2510        2520        2530        2540        2550
CACATATAAT ACAATCTCCA TGAAAAGATC CGCTAGACGA GCACAAATAT

      2560        2570        2580        2590        2600
ATACTTTTTA TTAAAAACGG AGGTCATTTC AATACCTATT GAAGAAATAA

      2610        2620        2630        2640        2650
ATTTTTTTTT TTTTTTTTTT TGTCATGACA CTTTTTTTTT TTTGTCATGA

      2660        2670        2680        2690        2700
CAGAATTGAA AAAAACAGAA AGTTATATAT TTACCCCCTT TAATTTTTTT
```

Fig. 15-4

```
      2710        2720              ClaI      2740        2750
TAÄAACTTTT GAAACTTTAG TAATAAGATC GATCTATACT TCAGTACGAA

      2760        2770        2780        2790        2800
CATAAATATG TATAAACCAA AAAAATTGAT TAAGATAAAG TTATATGTTT

      2810        2820        2830        2840        2850
GTATTTAATA AAATAGTTTA GTTTAAAATT TTATATCATT TTTTAAAAAA

      2860        2870        2880        2890        2900
TGAAAATGTT TGAAAAAAAA AATTTTTTTT TTTTTTTTCA ACGGGACGAT

      2910        2920        2930        2940        2950
GTAATATCAT ATATGATTCA AAATTAAAAG TTATTAACAA ATATGTAAAA

      2960        2970        2980        2990        3000
ATTATAAAAA ACTAACCTAG TTATAATTAC TTTCCCCTCT TTTTTTTTTT

      3010        3020        3030        3040        3050
TTTTTTTGTC ATGACACTTT TTTTTTTTTG TCATGACACT TTTTTTTTAA

      3060        3070        3080        3090        3100
AAAAAAAAAA AAAAATGTTA AAATACTATT TGATGACATT CATTTTTCCT

      3110        3120        3130
AGTTTTTTTT TAGATAGATA TAAAAATAAA TTGCCTAT
```

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 15/11, C12N 15/79, 15/80, C07K 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	DERWENT DATA BASES : WPAT, USPA, BIOTECHNOLOGY KEYWORDS : DICTYOSTELIUM

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

AUST CLASS as above
CHEM ABS using Keywords above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X Y	Plasmid Vol 20 (1988) (Barbara Leiting and Angelika Noegel) "Construcxtion of an Extrachromosomally replicating transformation vector for Dictyostelium discoideum" pp 241-248. Whole document	(1,2,5,6,14) (17,22-24)
Y	Proc. Natl. Acad. Sci. USA, Vol 86 October 1989 Joseph L. Dynes and Richard A. Firtel (Molecular complementation of a genetic marker in Dictyostelium using a genomic library" pp 7966-7970, see page 7966 line 1-71 and page 7969 2nd paragraph - page 7970.	(1,2,5,6,14, 17,22-24)
A	Gene, Vol 39, (1985) Wolfgang Nellen & Richard A. Firtel "High copy number transformants & co transformation in Dictyostelium" pp 155-163.	

(continued)

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8 February 1991 (08.02.91))	20 February 1991
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	A.W. BESTOW

A	The Embo Journal Vol 2 No.4 (1983) Metz et al "Identification of an endoyenous plasmid in Dictyostelium discoideum" pp 515-519.	

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Auth rity did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No prot st accompanied the payment of additional search fees.